The Prevention of Heparanase Expression in Endothelial Cells Injured by High Glucose

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PREFACE

This thesis has been organised as a series of manuscripts that will be submitted for publication in scientific journals. Some repetition of introductory and methodological material is unavoidable.

ABSTRACT

Vascular complications, in microvessels resulting in nephropathy, retinopathy and neuropathy and in macrovessels resulting in atherosclerosis caused by hyperglycemia contribute greatly to the morbidity and mortality in diabetes mellitus. In the vasculature, the endothelial cells (ECs) are first to be damaged by hyperglycemia due to their unique location as the inner lining of all vessels. There are several mechanisms involved in endothelial injury or dysfunction, however, the degradation of heparan sulfate proteoglycan (HSPG) on the cell surface and in the extra cellular matrix (ECM) is considered to be of importance. Heparanase is believed to degrade heparan sulfate (HS). Our objectives were to determine if heparanase is responsible for endothelial injury and dysfunction in diabetes.

To determine if hyperglycemia and heparanase cause endothelial injury, high concentrations of glucose (30mM), mimicking hyperglycemia and optimal doses of heparinase I were used to treat cultured porcine aortic endothelial cells (PAECs). Cell injury was measured by determining live cell number and lactate dehydrogenase (LDH) release. To determine if heparanase is expressed in high glucose treated PAECs, reverse transcriptase polymerase chain reaction (RT-PCR) was used to amplify heparanase mRNA. In addition, heparanase activity was measured by incubating cell lysates with ^{35}S-labelled ECM from cultured bovine corneal ECs, where released radioactive HS was analyzed by Sepharose gel filtration followed by β-scintillation counting. To help understand

the mechanism of high glucose injury, heparanase mRNA and activity were also measured in PAECs treated with H_2O_2 or mannitol to determine if free radical injury or osmolarity caused effects similar to high glucose treatment. As well, high glucose or heparinase I treated PAECs were also treated with heparin (0.5 µg/ml) and/or insulin (1 U/ml) and/or basic fibroblast growth factor (bFGF, 1 ng/ml) to determine if these compounds protected ECs from injury or inhibited heparanase expression induced by high glucose.

PAECs injured by high glucose or heparinase I (0.3 U/ml in serum free medium) showed a significantly decreased live cell number and increased LDH release compared to control cells. High glucose or heparinase I treated ECs showed an increase in live cell number and decrease in LDH release when treated with heparin and/or insulin and bFGF. Heparanase mRNA and activity was expressed in PAECs treated with high glucose or H_2O_2. Heparin and/or insulin, but not bFGF prevented heparanase mRNA expression and activity in high glucose treated PAECs. Mannitol did not induce the upregulation of heparanase mRNA and activity. bFGF showed variable protection in cells treated with high glucose or heparinase I when combined with insulin or heparin.

From these results we conclude that hyperglycemia is a main cause of endothelial injury. Heparanase production induced by hyperglycemia is responsible for EC injury and vascular dysfunction likely through the degradation of HS, resulting in increased vascular permeability and detachment of cells from the basement membrane. The mechanism of heparanase upregulation may be related to the formation of reactive oxygen species, but not

due to changes in osmolarity. Heparin and/or insulin and bFGF protect cells from injury caused by high glucose or heparinase I. Heparin and/or insulin but not bFGF inhibit heparanase mRNA upregulation induced by high glucose. This study provides new insight into the causes of vascular injury associated with diabetes and suggests possible treatments to reduce endothelial injury.

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DEDICATION

This thesis is written in dedication to my parents, Qinghua Han and Yunfeng Yang. They have always been very supportive all my life. They even did not inform me of my mom's major operation in China, thinking it would add an emotional burden to me when I am completing my studies. Thanks, Mom.

TABLE OF CONTENTS

PERMISSION TO USE ……i

PREFACE ……ii

ABSTRACT……..iii

ACKNOWLEDGEMENT……vi

DEDICATION ……vii

TABLE OF CONTENTS ……..viii

LIST OF FIGURES……x

LIST OF ABBREVIATIONS ……xii

1. LITERATURE REVIEW

1.1. Diabetes……....1

1.1.1. Definition of Diabetes……1

1.1.2. Vascular Damage due to Hyperglycemia……..2

1.1.3. Insulin and Diabetes……9

1.1.4. Complications of Diabetes……11

1.1.5. Morbidity and Mortality of Diabetes……..15

1.2. Endothelial Dysfunction in Diabetes……...16

1.2.1. Function of Endothelium……16

1.2.2. The Concept of Endothelial Dysfunction……..18

1.2.3. Endothelial Dysfunction Associated with Hyperglycemia……18

1.3. Degradation of Heparan Sulfate Proteoglycan (HSPG)……...19

1.3.1. Proteoglycans……..19

1.3.2. Heparan Sulfate Proteoglycans....……20

1.3.3. A Unique Heparan Sulfate: Heparin ……22

1.3.4. Effect of HSPG Degradation ……25

1.3.5. Basic Fibroblast Growth Factor (bFGF) Released by HSPG Degradation ……26

1.4. Heparanase ……...28

1.4.1. Properties of Heparanase ……28

1.4.2. Pathogenesis Caused by Heparanase ……...33

1.4.3. Heparanase in Diabetes Mellitus ……34

1.5. Summary……...35

2. OBJECTIVES ……..38

3. Protection of Porcine Aortic Endothelial Cell Injury from High Glucose by Insulin, Heparin and bFGF

3.1. Introduction …….....40

3.2. Materials and Methods……...46

3.3. Results …….....51

3.4. Discussion ……....67

4. Heparanase Upregulation in High Glucose Treated Porcine Aortic Endothelial Cells is Prevented by Insulin and Heparin

4.1. Introduction …….....77

4.2. Experimental Procedures ……79

4.3. Results…….....86

4.4. Discussion……...96

5. GENERAL DISCUSSION……...102

6. CONCLUSIONS…….....108

7. REFERENCES…….....110

LIST OF FIGURES

1.1. Polyol Pathway……5

1.2. The Aldose Reductase Pathway of Glucose Metabolism……6

1.3. Formation of Advanced Glycosylation
End Products from Glucose……..8

1.4. Major and Variable Sequences of Original and Fully
Sulfated Heparin and Heparan Sulfate……..24

1.5. Cleavage of HSPG by Heparanase...……30

1.6. Scheme of the Human Heparanase Gene and Protein……..32

3.1. PAECs Injured by High Glucose were
Protected by a Combination of Heparin and Insulin……....53

3.2. The Protective Effect of Insulin and/or
Heparin on PAECs Injured by High Glucose……55

3.3. Insulin and/or Heparin Protected PAECs
from High Glucose Injury when bFGF was
Present in Cell Medium……...57

3.4. Heparinase I Dose Response Shown in PAECs Cultured
in M199 with Serum when Treated for Six Days……..59

3.5. Heparinase I Dose Response Shown in PAECs Cultured
in M199 with Serum when Treated for Ten Days…….....61

3.6. Heparinase I Dose Response Shown in PAECs
Cultured in Serum Free M199……62

3.7. Heparinase I Induced PAECs Injury was Prevented
by Insulin and/or Heparin……64

3.8. The Protective Effect of Insulin and/or Heparin
on PAECs Injured by Heparinase I when
bFGF was Present in Cell Medium……...66

4.1. Heparanase mRNA was not detectable in Fresh
Untreated Porcine Tissue……...88

LIST OF FIGURES CONTINUED

4.2. Heparanase mRNA was not detectable in Primary Non-Confluent and Confluent Cultured PAECs……...89

4.3. High Glucose Induced Heparanase mRNA which was Inhibited by Insulin and/or Heparin…….90

4.4. Heparanase mRNA was Induced by H_2O_2, but not Mannitol……91

4.5. Heparanase mRNA in High Glucose and bFGF Treated PAECs……..92

4.6. High Glucose Induced Heparanase Activity which was Inhibited by Insulin and/or Heparin……...94

4.7. Heparanase Activity was present in H_2O_2 but not in Mannitol Treated PAECs……...95

LIST OF ABBREVIATIONS

ACE	angiotension-converting enzyme
AGE(s)	advanced glycosylation end-product(s)
BCEC	bovine corneal endothelial cell
bFGF	basic fibroblast growth factor
BM	basement membrane
cGMP	cyclic 3'-5'- guanosine monophosphate
CHO	chinese hamster ovary
CMF-DPBS	calcium- magnesium- free Dulbecco's phosphate-buffered saline
CTAP-III	connective tissue activating peptide III
DAG	diacylglycerol
DM	diabetes mellitus
DMSO	dimethyl sulfoxide
ECM	extracellular matrix
EC(s)	endothelial cell(s)
EDNO	endothelial-derived nitric oxide
ET-1	endothelin-1
FBS	fetal bovine serum
FGF	fibroblast growth factor
FGFR	FGF receptor
GAG(s)	glycosaminoglycan(s)
GBM	glomerular basement membrane
HGF	hepatocyte growth factor
HIP	HS/heparin-interacting protein
HS	heparan sulfate
HSPG(s)	heparan sulphate proteoglycan(s)
Il-1	interleukin-1
LDH	lactate dehydrogenase
NCP	neutrophil cationic protein
NO	nitric oxide
NOS	nitric oxide synthase
PAEC(s)	Porcine aortic endothelial cell(s)
PAI-1	plasminogen activator inhibitor-1
PGI_2	prostaglandin I_2
PG(s)	proteoglycan(s)
PI	phosphoinositides
PKC	protein kinase C
ROS	reactive oxygen species
RT-PCR	reverse transcriptase polymerase chain reaction
TGF-β	transforming growth factor-*beta*

LIST OF ABBREVIATIONS CONTINUED

TNF-α	tumor necrosis factor-α
tPA	tissue plasminogen activator
VEGF	vascular endothelial cell growth factor
VSMC	vascular smooth muscle cell
vWF	von Willebrand Factor

1. LITERATURE REVIEW

1.1. Diabetes

1.1.1. Definition of Diabetes

Diabetes is defined as a disorder of carbohydrate metabolism. The most common form of diabetes is diabetes mellitus (DM) in which there is an inability to oxidize carbohydrate due to disturbances in insulin function. Insulin is the principal hormone released by pancreatic β-cells that regulates uptake of glucose into cells (primarily muscle and fat cells) from the blood. Insulin makes it possible for most body tissues to remove glucose from the blood for use as fuel, for conversion to other needed molecules, or for storage. Insulin is also the principal control signal for conversion of glucose to glycogen for storage in liver and muscle cells. If the amount of insulin produced is insufficient, if cells respond poorly to the effects of insulin (insulin insensitivity or resistance), or if the insulin itself is defective, glucose is not handled properly by body cells nor stored appropriately in the liver and muscle. The net effect is persistent high levels of blood glucose, poor protein synthesis, and other metabolic derangements. Therefore, diabetes mellitus is considered to be a group of diseases characterized by hyperglycemia resulting from defects in insulin production, insulin action, or both. Diabetes

mellitus is typically classified into two main subtypes: type 1 and type 2. Type 1 diabetes develops when the pancreatic *β*-cells are destroyed by the body's autoimmune system and can not produce the insulin that regulates blood glucose. Type 2 diabetes usually begins as insulin resistance, a disorder in which the cells do not use insulin properly. As the need for insulin rises, the pancreas gradually loses its ability to produce insulin. Type 1 diabetes usually strikes children and young adults and may account for five to ten percent of all diagnoses of diabetes. Type 2 diabetes is associated with old age, obesity, family history of diabetes, prior history of gestational diabetes, impaired glucose tolerance, physical inactivity, and race/ethnicity and may account for 90 to 95 percent of all diagnosed cases of diabetes. Type 2 diabetes is increasely being diagnosed in children and adolescents (National Diabetes Statistics 2002).

1.1.2. Vascular Damage due to Hyperglycemia

Hyperglycemia is considered to be one of the most important predisposing factors in the development of diabetic complications (Ruderman *et al*., 1992; Lee *et al*., 1989a; Kamata *et al*., 1992; Natarajan *et al*., 1992). The evidence linking hyperglycemia and microvascular disease in diabetes was obtained in clinical studies. The patients under better glycemic control developed fewer eye and/or renal complications (Pirar ， 1977). Hyperglycemia causes cell damage which ultimately leads to irreversible structural abnormalities characterized by loss of cells and progressive

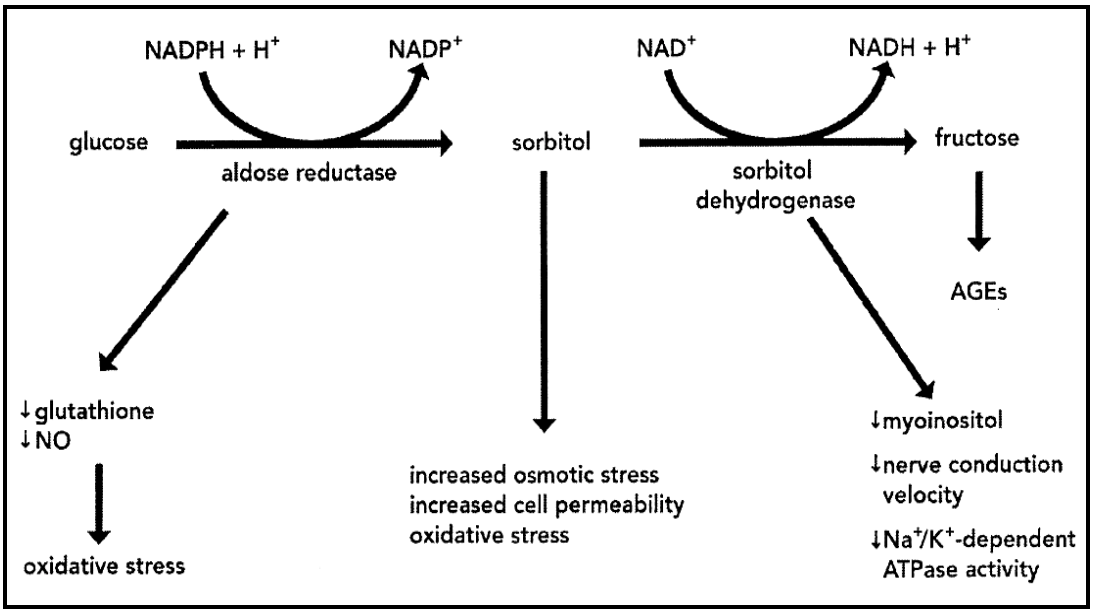
microvascular occlusion in the eye and kidney, and by intimal proliferation and occlusion in large vessels (Kohner *et al*., 1982; Bresnick *et al*., 1997; Steffes *et al*., 1989; Osterby, 1990). Hyperglycemia induced microvascular hypertension also contributes directly to an increase in vascular permeability, and the resultant extravasations of plasma proteins and growth factors, both in small and large vessels, may promote irreversible hypertension and vessel occlusion. Additional evidence is derived from studies showing that hyperglycemia associated with diabetes produce many of the metabolic, biochemical, and functional abnormalities seen in the vasculature. At least four different mechanisms have been postulated to contribute to these abnormalities.

The first mechanism which may contribute to diabetic complications is an increase in the formation of sorbitol (Tilton *et al*., 1989; Pugliese *et al*., 1990; Bank *et al*., 1989). In the hyperglycemic state, excess glucose entering the polyol pathway can lead to the accumulation of sorbitol by aldose reductase coupled with oxidation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) to its oxidized form $NADP^+$. Sorbitol can be subsequently converted to fructose by sorbitol dehydrogenase coupled with the reduction of oxidized nicotinamide-adenine dinucleotide (NAD^+) to the reduced form NADH as shown in Figure 1.1. Aldose reductase is ubiquitous and therefore polyols accumulate in nearly all tissues of diabetic patients. Increased flux through this pathway due to hyperglycemia, increases intracellular sorbitol and fructose, increases osmotic pressure, decreases

myoinositol concentration, and in combination with glycolysis, alters the intracellular redox balance. NADPH is required for nitric oxide (NO) regeneration and replenishes the antioxidant glutathione. Thus, increased cytosolic NADH/NAD^+ ratio decreases NADPH resulting in the formation of intracellular reactive oxygen species (ROS), leading to oxidative stress (Brownlee, 2001). Certain abnormalities in the microvasculature of diabetic rats are prevented by administration of an inhibitor of aldose reductase (Beyer-Mears *et al*., 1986; Craven and DeRubertis, 1989a; Cohen, 1986; Robison *et al*., 1989).

The second mechanism is decreased myoinositol-dependent Na/K-ATPase as shown in Figure 1.2. Myoinositol is a normal constituent of the diet and is also synthesized by many cells (Greene *et al*., 1987). It plays an important role in signal transduction and the synthesis of phosphoinositides (PI). Adenosine-mediated hydrolysis of PI leads to the release of a mediator that activates Na/K-ATPase and thereby regulates many cell functions. Depletion of the intracellular pool of PI caused by a sorbitol pathway-linked transport defect and a decrease in its response to adenosine both occur in diabetes, which can be prevented by aldose reductase inhibitors (Beyer-Mears *et al*., 1986; Robison *et al*., 1989; Winegrad， 1987).

The third mechanism is increased synthesis of diacylglycerol (DAG) with the consequent activation of several isoforms of protein kinase C (PKC) (Lee *et al*., 1989a; Craven and DeRubertis 1989b; Williamson *et al*., 1990).



(© Setter *et al*. 2003. Figure prepared by Dr. William Light on behalf of Medi Tech Media Ltd)

Figure 1. 1. Polyol Pathway

Activation of the polyol pathway results in a decrease of reduced $NADP^+$ and oxidized NAD+; these are necessary cofactors in redox reactions throughout the body. The reduction in these cofactors leads to decreased levels of reduced glutathione, NO and myoinositol.

AGEs: advanced glycation end-products

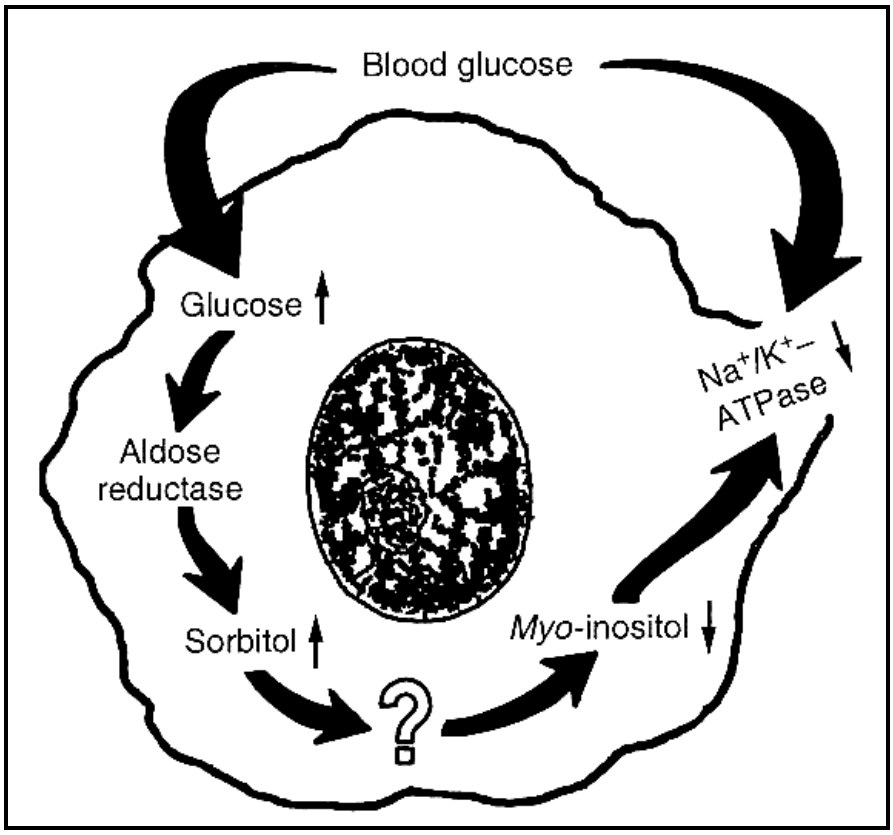
NAD: nicotinamide-adenine dinucleotide

NADH: reduced nicotinamide-adenine dinucleotide

NADPH: nicotinamide-adenine dinucleotide phosphate

$NADPH^+$: oxidized nicotinamide-adenine dinucleotide phosphate

NO: nitric oxide



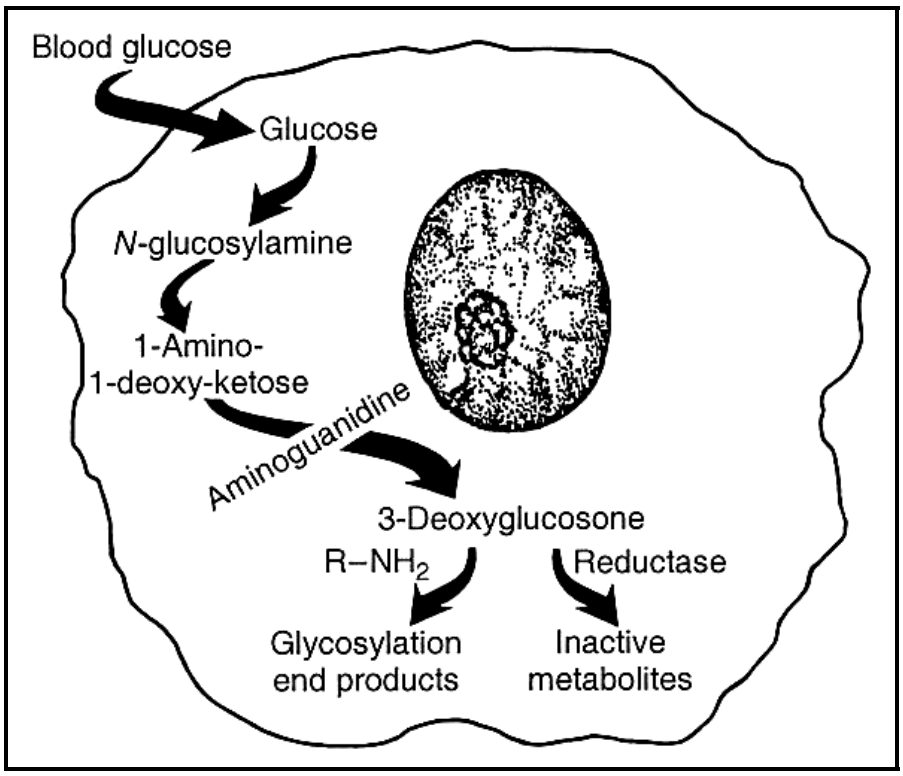
(Clark and Lee, 1995)

Figure 1.2. The Aldose Reductase Pathway of Glucose Metabolism

The aldose reductase pathway is activated by intracellular hyperglycemia, resulting in increase sorbitol formation. This, in turn (through an unknown mechanism, indicated by the question mark), results in decreased myo-inositol formation and ultimately in decreased cellular activity of Na^+K^+-ATPase. Hyperglycemia also directly inhibits ATPase activity. The vertical arrows indicate increases (↑) and decreases (↓) in the substances in question.

Several studies have shown that hyperglycemia increases DAG concentration in several types of vascular cells and tissues and causes an activation of PKC, probably by its translocation from cytosol to the membrane (Lee *et al*., 1989a and Lee *et al*., 1989b). PKC is probably activated by DAG synthesized by means of a stepwise acylation of glycerol 3-phosphate generated as a by-product of glycolysis. Increased DAG levels and activation of PKC have been linked to cell growth, permeability, contractility and synthesis of extracellular matrix protein resulting in the abnormal vascular function associated with diabetes (Williamson *et al*., 1990).

The fourth mechanism is covalent modification of protein by nonenzymatic glycosylation. Nonenzymatic glycosylation has been shown to result in qualitative and quantitative changes in extracellular components that can affect cell adhesion, growth, and matrix production (Tsilibaru *et al*., 1988; Charonis *et al*., 1990; Sensi *et al*., 1989). Many of these effects are mediated by advanced glycosylation end-products (AGEs), which form from 1-amino-1-deoxyketose adducts through a complex series of dehydration, rearrangements and redox reactions (Monnier and Cerami， 1983) as described in Figure 1.3. Matrix accumulation of AGEs may further accelerate diabetic vascular occlusion by blunting the effect of the vasodilatory factor NO, which is quenched by AGEs in a dose-dependent fashion (Bucala *et al*., 1991).



(Clark and Lee, 1995)

Figure 1.3. Formation of Advanced Glycosylation End Products from Glucose

The formation of advanced glycosylation end products from glucose occurs through the nonenzymatic formation of early glycosylation products (N-glucosylamine) that then undergo acid-base catalysis to form Amadori products (1-amino-1-deoxy-ketose). Advanced glycosylation end products result from the degradation of the Amadori products into reactive carbonyl compounds that react with free amino groups (R-NH_2). The formation of advanced glycosylation end products *in vivo* is retarded by reductase.

Alterations in four such interrelated hyperglycemia-driven pathways have been linked to the early microvascular changes associated with diabetes in animal and human tissues (Williamson *et al*., 1988; Walker and Viberti， 1991). Hyperglycemia can also contribute directly to microvascular occlusion by stimulating extracellular matrix synthesis (Brownlee and Spiro, 1979; Moran *et al*., 1991; Mandal *et al*., 1995). Various abnormalities, such as AGEs, PKC and cytokines such as transforming growth factor-*beta* (TGF-*β*), and increased oxidative stress in diabetes contribute to synthesis of extracellular matrix proteins, i.e. type IV collagen, laminin and fibronectin. In addition, loss of cells may indirectly contribute to further microvascular occlusion by stimulating excessive production of extracellular matrix (ECM) (McNeil *et al*., 1989; Okuda *et al*., 1990; Saito *et al*., 1988).

1.1.3. Insulin and Diabetes

Insulin, a circulatory hormone produced in response to elevated glucose concentration in the blood, regulates the metabolism of glucose. Glucose is liberated from dietary carbohydrate such as starch or sucrose by hydrolysis within the small intestine, and is then absorbed into the blood. Elevated concentrations of glucose in blood stimulate synthesis and release of insulin from pancreatic *β*-cells. Insulin acts on cells throughout the body to stimulate uptake, utilization and storage of glucose. Besides its role in regulating glucose metabolism, insulin stimulates lipogenesis, diminishes lipolysis, and increases amino acid transport into cells. Insulin also

modulates transcription, altering cell content of numerous mRNAs. It stimulates growth, DNA synthesis, and cell replication, effects that it holds in common with insulin-like growth factors (Sowers, 1997; Bornfeldt *et al*., 1992).

The vasculature is an insulin-responsive tissue. Increased insulin concentration and decreased vasculature sensitivity to insulin have been identified as independent risk factors for cardiovascular disease in insulin-resistance syndromes and type 2 diabetes (Despres *et al*., 1996; Howard *et al*., 1996). The activation of insulin receptors, by insulin binding, results in diverse signaling processes that mediate the many actions of insulin in vascular cells. Actions of insulin include the regulation of cell growth, gene expression, and protein synthesis. Studies of cultured vascular cells in the presence of insulin showed increasing cell mitogenosis, protein synthesis and production of matrix proteins. These changes can result in atherogenesis through vascular smooth muscle cell (VSMC) hypertrophy and hyperplasia, and extracellular matrix protein synthesis (Feener and King, 1997; Howard *et al*., 1996). However, endothelial-derived NO can reduce the progression of atherosclerosis through inhibiting proliferation of VSMC and platelet adhesiveness (Radomski *et al*., 1987; Moncada *et al*., 1988; Mollace *et al*., 1991). The ability of insulin to increase production of NO has been shown in cultured endothelial cells (ECs) (Zeng and Quon, 1996). NO is an important vasodilator and plays a critical role in the regulation of vascular tone and blood pressure (Vallance *et al*., 1989). In contrast, it has been shown that a

high dose of insulin increases endothelin-1 (ET-1) levels in cultured ECs (Metsarinne *et al*., 1994; Ferri *et al*., 1995a; Mandal *et al*., 2000). Increased ET-1 levels are also found in type 2 diabetic subjects in response to hyperinsulinemia (Ferri *et al*., 1995a; 1995b). ET-1 exerts a potent vasoconstrictor effect on the vasculature and produces an elevation of systemic blood pressure in anesthetized dogs and rats (Yokoawa *et al*., 1989; Miller *et al*., 1989). Some studies showed that hypertensive patients have high ET-1 levels in their plasma (Shichiri *et al*., 1990; Kohno *et al*., 1990) and patients with endothelin dependent malignant hemangioendothelioma have hypertension induced by an elevated level of plasma ET-1 (Yokoawa *et al*., 1991). The actions of insulin appear to be contrary regarding the production of both of NO and ET-1. Reports show the ability of insulin to induce vasodilatation is low in insulin resistance and diabetes (Steinberg *et al*., 1996) which could be due to inactivation of NO or the inability of ECs to produce NO (Ting *et al*., 1996). Thus, insulin may have antihypertensive and anti-atherogenic actions at an appropriate physiological dose or in the non-diabetic state.

1.1.4 Complications of Diabetes

Diabetes mellitus resulting from defects in insulin production, insulin action, or both is characterized by hyperglycemia and abnormal lipid and protein metabolism. These changes are associated with diabetic

complications, especially cardiovascular diseases including microvascular disease, macrovascular disease and diabetic cardiomyopathy.

Diabetic microvascular disease affects the small vessels such as those present in the retina, near nerves and in the microvasculature of the kidneys. This systemic disease in diabetes clinically leads to retinopathy and glomerular dysfunction, and possibly contributes to neuropathy. A number of studies have suggested that hyperglycemia appears to be the central initiating factor for all types of diabetic microvascular diseases (Ruderrman *et al*., 1992; Kamata *et al*., 1992). Some evidence derived from animal studies of experimental diabetes with hyperglycemia also showed lesions in the eye (Engerman and Kern, 1984) and kidney (Engerman and Kern, 1989; Rasch, 1981) similar to those in humans. Patients under better glycemic control developed fewer eye and/or renal complications in a clinical study (Pirart, 1977). Non-proliferation diabetic retinopathy is associated with pericyte loss, formation of microaneurysms, increased vascular permeability, and capillary closure that can lead to areas of non-perfusion and ischemia. The retina responds to this hypoxia by increased production of vascular endothelial cell growth factor (VEGF), which promotes neovascularisation (Aiello *et al*., 1994). In diabetic nephropathy, an increase in both intraglomerular pressure and extracellular matrix proteins results in basement membrane thickening, mesangial expansion, glomerular hypertrophy and narrowing of the lumen of the capillaries (Osterby, 1990). These changes reduce glomerular filtration area and function and impede blood flow, and can progress to

glomerulosclerosis (Mogensen *et al*., 1988), resulting in increasing albumin filtration rate in the glomerulus in microalbuminuric patients (Deckert *et al*., 1989). Diabetic neuropathy refers to a group of diseases that affect the peripheral nerves which extend outside the brain and spinal cord and include three types: motor, sensory and autonomic nerves. The autonomic nerves, not consciously controlled, supply the heart, blood vessel, bladder and intestinal tract. Diabetic neuropathy is classified as either peripheral (including sensor and motor) or autonomic, with peripheral neuropathy being the most common manifestation. Diabetic neuropathy results from both chronic and acute elevations in blood glucose. Decreased nerve function by acute hyperglycemia and reduced nerve fibers and nerve fiber reproduction by chronic hyperglycemia, are believed to occur because of the formation of sorbitol and advanced glycosylation end products (Clark and Lee, 1995) which cause microangiopathy, causing altered neuronal capillary flow (Koda-Kimble *et al*., 1995).

Macrovascular complications in diabetes, including coronary artery disease, peripheral vascular disease and cerebrovascular disease, is the result of an acceleration of atherosclerosis and increased thrombosis. Although the relationship between hyperglycemia and macrovascular disease in patients with diabetes is complicated by many other factors that influence atherogenesis in non-diabetics, an association between asymptomatic hyperglycemia and coronary heart disease has been found in both middle aged and elderly people (Jarrett *et al*., 1982; Mykkanen *et al*.,

1990). Many factors, some of which may exist in the prediabetic state, particular in type 2 diabetes, are involved in the etiology of macrovascular disease. Hyperlipidaemia, hypertension, hyperinsulinaemia and decreased insulin sensitivity related to the insulin-resistance syndrome have been proposed to induce atherogenesis even before the clinical diagnosis of type 2 diabetes (Haffner *et al*., 1990). In addition, diabetes is associated with coagulopathy and endothelial dysfunction with impaired NO production, compounding the effects of hyperglycemia in promoting atherogenesis (Jokl and Colwell, 1997). A number of factors, such as hyperinsulinaemia, insulin precursors, and tumor necrosis factor-α (TNF-α) are thought to increase plasma plasminogen activator inhibitor-1(PAI-1) concentrations. Local changes in vascular PAI-1 expression, which is increased in the neointima of atherosclerotic lesions, may also have an impact on the fibrinolytic balance in injured vessels. High PAI-1 levels inhibit fibrinolysis and facilitate the persistence of fibrin, which may damage the endothelium (Lupu *et al*., 1993). Endothelial dysfunction may reduce the antithrombotic properties of the endothelium by decreasing the synthesis of prostaglandin I_2 (PGI_2) and diminishing the endothelial protective effects against platelet adhesion (Wu and Thiagarajan, 1996). The common features in diabetic vascular disease, due to development of chronic hyperglycemia, are exaggerated proliferation of endothelium and thickening of the basement membrane (Fischer *et al*., 1979; Osterby, 1990; Kefalides, 1981) which result in narrowing of the blood

vessel lumen causing thrombosis, ischemia, and ultimately infarction (Colwell and Lopes-Virella, 1988).

Diabetic cardiomyopathy is characterized by cardiac contractile dysfunction and congestive heart failure due to myocellular hypertrophy and myocardial fibrosis (Bell, 1995). This induces abnormal left ventricular filling suggesting poor compliance or prolongation of left ventricular relaxation. The exact cause of this ventricular dysfunction is not known, but several mechanisms have been proposed, including (1) metabolic abnormalities of glucose transport; (2) increased extracellular matrix in the interstitium of the ventricular wall showed by increased type IV collagen and a combination of nonenzymatic glycation and enzymatic O-linked glycosylation. This may be the result of increased 2 N-acetylglucosaminyl transferase, that is increased in diabetic rat hearts (Nishio *et al*., 1995); (3) abnormalities in fatty-acid metabolism and alteration of calcium uptake by the sarcoplasmic reticulum leading to cellular calcium overload. These changes can result in stiffer ventricular walls and impaired myocyte contractility (Mizushige *et al*., 2000; Tahiliani and McNeill, 1986). In addition, coronary heart disease may make a large contribution to heart failure in diabetic cardiomyopathy.

1.1.5. Morbidity and Mortality of Diabetes

Cardiovascular disease is the major burden of diabetes mellitus. Both macrovascular and microvascular disease contribute greatly to the morbidity and mortality in diabetes (Kassab *et al*., 2001). Diabetes is associated with

accelerated atherosclerotic macrovascular disease affecting arteries that supply the heart, brain and lower extremities. As a result, patients with diabetes have much higher risk of myocardial infarction, stroke, and limb amputation. Up to 80% of deaths in people with type 2 diabetes are attributed to cardiovascular disease and stroke (Hanefeld *et al*., 1996). It is well established that chronic hyperglycemia results in microvascular complications in diabetes which is the leading cause of renal failure and blindness, contributing to more than 33,000 new cases of end-stage renal disease (United States Renal Data System, 2002) and more than 24,000 new cases of vision loss each year in the US (Diabetes 2001 vital statistics). According to the statistics of the American Diabetes Association (2002), the total economic cost including direct costs (cost of medical care and services) and indirect costs (cost of short term and permanent disability and premature death) is about 132 billion.

1.2 Endothelial Dysfunction in Diabetes

1.2.1. Function of Endothelium

The endothelium is a distributed organ consisting of a monolayer of metabolically active cells lining all blood and lymphatic vessels. It plays an important role in the structural integrity and normal function of the circulatory system. The vascular endothelium has extensive regulatory capacities and senses changes in hemodynamic forces and blood borne signals to release a number of vasoactive substances which are critical to maintain vascular homeostasis.

Endothelium acts as a selective permeability barrier for substances including H_2O, CO_2, ions, small lipid molecules, and plasma proteins for exchange between the blood and extravascular tissue, maintaining the homeostatic balance between the intra and extra cellular environment.

Endothelial cells normally inhibit platelet adhesion and aggregation by producing PGI_2 and nitric oxide (NO). Endothelial cells limit activation of the coagulation cascade by the thrombomodulin-protein C and the heparan sulfate-antithrombin III pathway, and regulate fibrinolysis by producing tissue plasminogen activator (tPA) and its inhibitor-1 (PAI-1), and thus maintain blood fluidity (Borsum, 1991; Pearson, 1991).

Endothelium plays a role in metabolism by displaying enzymes responsible for a number of important biochemicals reactions including angiotensin converting enzyme (ACE) which is an ectoenzyme found on the luminal surface of the endothelium. The function of ACE is to convert angiotensin I to angiotensin II, a vasopressor (Webb and Cockcroft, 1990).

Endothelial cells secrete multiple vasoactive substances critical in the regulation of vascular tone as well as a wide array of cytokines and growth factors such as transforming growth factor-beta (TGF-β) and angiotensin II. Endothelium affects vascular smooth muscle and mensangial cell function by producing mediators such as NO and endothelin-1. Endothelin-1 stimulates contraction and proliferation of smooth muscle and mesangial cells while NO has opposite effects (Cohen and Tesfamariam, 1992; Stehouwer, 1998).

1.2.2. The Concept of Endothelial Dysfunction

The vascular endothelium is not a mere barrier between intravascular and interstitial compartments, but a widely spread organ, which is responsible for the regulation of hemodynamics, angiogenic vascular remodeling, and metabolic, synthetic, inflammatory, antithrombogenic, and prothrombogenic processes. As any other organ, the vascular endothelium is a subject for dysregulation, dysfunction, insufficiency, and failure. Endothelial dysfunction describes disturbances in the barrier function of the vascular endothelium, impaired antithrombogenic properties; perturbed angiogenic capacity; inappropriate regulation of vascular smooth muscle tonicity, proliferative capacity, migratory properties; perturbed synthetic functions; and prevention of neutrophils and monocytes from diapedesis.

1.2.3. Endothelial Dysfunction Associated with Hyperglycemia

The vascular endothelium is the initial target site of injury in diabetes. The proximate causes of endothelial injury are not known but likely include hyperglycemia, exporsure to advanced glycosylation end-products, activation of protein kinase C, increased expression of TGF-*β* and vascular endothelial growth factor (VEGF), oxidative stress and other components of the insulin resistance syndrome which are hyperinsulinaemia, hypertension and dyslipidaemia (Stehouwer *et al*., 1997). These factors probably contribute to endothelial dysfunction in human diabetic complications and include the following: the endothelium loses its antithrombotic and profibrinolytic nature

and may instead acquire prothrombotic and antifibrinolytic properties, a transition marked by high plasma levels of thrombomodulin and PAI-1. Platelet adhesion and aggregation are no longer inhibited but may actually be stimulated by prethrombotic factors released from injured endothelial cells, such as Tissue Factor and von Willebrand Factor (vWF) (Stehouwer, 1998). Endothelium-dependent, nitric oxide-mediated vasodilatation decreases and vascular smooth muscle cell contraction is enhanced so hypertension ensues (Elliott *et al*., 1993). One of the most important forms of endothelial dysfunction is basement membrane thickening and an increase in vascular permeability which may result from loss of heparan sulphate proteoglycan (HSPG) (Kanwar *et al*., 1980). The glomerular filter is compromised and an increased albumin filtration rate in the glomerulus is observed in microalbuminuric patients (Deckert *et al*., 1989).

1.3. Degradation of Heparan Sulfate Proteoglycan (HSPG)

1.3.1. Proteoglycans

Proteoglycans (PGs) are ubiquitous macromolecules that are produced by most eukaryotic cells, and are found predominantly on the cell surface, in the ECM and intracellular granules (Kjellen and Lindahl, 1991). The structure of these PGs includes a core protein onto which are bound numerous sulfate and/or acetylated polysaccharide chain subunits called glycosaminoglycans (GAGs). PG structure begins with the core protein which may contain a few or many GAG binding sites which are substituted to

various degrees with side chain polysaccharides made up of repeating hexosamine and hexuronic acid subunits. These can include both acetylated and sulfated components organized in patterns which impart localized regions of high anionic charge alternating with neutral segments (Choay and Petitou, 1986). The relative arrangement of these charged and uncharged areas provides the molecule with additional information carrying ability. The unique structural features of PGs endow them with properties that may influence a wide range of biological processes. Several physically related GAGs including heparan sulfate (HS), dermatan sulfate, chondroitin sulfate, and hyaluronic acid have been identified in association with cell membranes and in the ECM (Hook *et al*., 1984) and play a major role in cell-cell and cell-protein interaction.

1.3.2. Heparan Sulfate Proteoglycans

HSPGs, found on cell membranes and in the ECM, are prominent components of blood vessels. In large vessels, they are concentrated mostly in the intima, where endothelial cells lie on their basement membrane (BM), and in the inner media which contains ECM including several elastic laminae. In capillaries they are found mainly in the subendothelial BM, where they support proliferation and migrating ECs and stabilize the structure of the capillary wall. The BM contains type IV collagen associated with laminins, entactin and HSPGs. HSPGs play an important role in the interaction between cell to cell and cell to ECM or BM.

The ECM is a meshwork-like substance found within the extracellular space and includes the BM of cells. The ECM not only provides structural support to cells and tissues, but also plays important roles in regulating the behavior of cells in multicellular organisms. The ECM is composed of a complex mixture of proteins and PGs including HSPGs. Almost all of the proteins are glycoproteins having short chains of carbohydrate residues attached to them. The proteins include a wide variety of collagens, laminins abundant in the basal lamina of epithelia, fibronectin binding cells to the ECM, and elastins providing flexibility to arteries, lungs, and skin. Fibronectin interacting with PGs, fibrin/fibrinogen and collagens are important for cell adhesion, growth and migration. The PGs promote adhesion of the cell to ECM through binding molecules such as fibronectin and laminin. Endothelial cells have been shown to synthesize the constituents of the ECM and control vascular permeability to macromolecules by modulating the biochemical and biophysical properties of the ECM (Chon *et al*., 1997; Borsum, 1991).

HSPGs like syndecans are also on the cell surface, and are believed to be the endogenous receptor for circulating growth factors i.e., basic fibroblast growth factor (bFGF), TGF-*β*, VEGF, hepatocyte growth factor (HGF) and chemokines that regulate cell proliferation, differentiation and migration (Linhardt and Toida, 1997). The ability of the HS side chains to interact with enzymes such as coagulation factors, mast cell proteases, lipoprotein lipase and cytokines such as granulocyte macrophage-colony

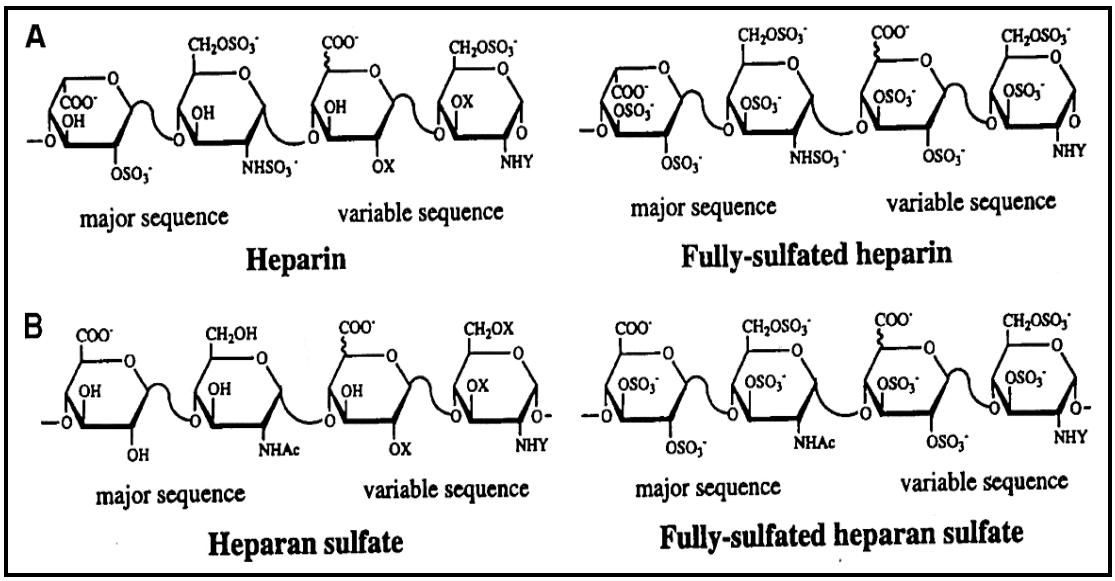
stimulating factor, interferon, interleukins also suggests an involvement in the control of normal and pathologic processes, such as wound healing, tumor growth and vessel formation (Vlodavsky *et al*., 1993). Hence, HSPGs play important roles in physiological processes of embryonic development, tissue repair, and regulation of blood coagulation, cartilage function and glomerular filtration (Kjellen and Lindahl, 1991; Bernfield *et al*., 1992; Salmivirta and Jalkanen, 1995). Several disease conditions such as cardiovascular diseases, cancer, inflammation, diabetes and amyloidosis are associated with changes in the expression of PGs as well as with structural and functional alterations of their GAG components (Rosenberg *et al*., 1997; Iozzo and Cohen, 1993; Jackson, 1997; van der Woude and van Det, 1997).

1.3.3. A Unique Heparan Sulfate: Heparin

Heparin is biosynthesized and stored in the “basophilic” granules of mast cells which are located in connective tissue, near the capillaries and in the walls of blood vessels, and is released as a GAG. Heparin is more highly sulfated, has high negative charge density and uronic acid is primarily iduronic acid versus glucouronic acid in heparan sulfate. Both heparin and HS have a high negative charge density, the result of sulfate and carboxylate residues present in their structure (Harpel *et al*., 1996) as described in Figure 1.4. These negative charges on the luminal surface of endothelium are thought to play an important role in controlling vascular permeability by acting as a “charge barrier” to the transvascular movement of the largely anionic

plasma protein such as albumin (Hardebo and Kahlstrom, 1985). Exogenous heparin is used as an anticoagulant and/or antithrombotic agent to maintain blood flow in the vasculature through the binding and activation of antithrombin III and other mechanisms (Harpel *et al*., 1996). Exogenous heparin is also capable of ameliorating increased vascular permeability caused by various polycationic substances including protamine, poly-L-lysine or neutrophil cationic protein (NCP) (Fairman *et al*., 1987; Peterson *et al*., 1987). Heparin is considered as a potent vasodilator that most likely lowers elevated blood pressure in hypertensive animal models by interacting with vascular endothelial cyclic 3'-5'- guanosine monophosphate (cGMP) or releasing endothelial derived NO (Mandal *et al*., 1995). NO may subsequently suppress ET-1 production by a cGMP-dependent pathway (Boulanger and Luscher, 1990) decrease VSMC contractility and increase vascular wall refractoriness to other vasoconstrictor substances (Mandal *et al*., 1995). In addition, heparin has antiviral activity, binds to a variety of growth factors, inhibits complement activation and regulates angiogenesis (Casu, 1985; Lane and Linhardt, 1989).

The ability of heparin to protect endothelium has been demonstrated by a number of studies: (1) Heparin administered by parenteral and oral routes accumulates within endothelium with its concentration hundreds to thousands-fold more than in plasma (Hiebert *et al*., 1993). (2) Heparin increases amounts of HS on EC surfaces (Nader *et al*., 1991). (3) Heparin is



(Garg *et al*., 2000)

Figure 1.4. Major and variable sequences of original and fully sulfated heparin (A) and heparan sulfate (B).

There are more negative charges in the sequences of fully sulfated versus original heparin and heparan sulfates

protective of cultured ECs damaged by exposure to free radicals (Hiebert and Liu, 1990).(4) Heparin in a time- and dose-dependent manner smoothes the endothelial surface and reduces actin and vimentin expression, shown by immunofluorescence microscopy (Mandal *et al*., 1995). (5) Heparin, insulin or a combination of insulin and heparin prevented EC intercellular gaps when cultured in high concentrations of D-glucose as viewed by scanning electron microscopy. In addition high glucose treated cultured VSMCs, showed enhanced actin expression which was blunted with addition of either heparin or insulin (Mandal *et al*., 2000).

1.3.4. Effect of HSPG Degradation

Depletion of HS and/or abnormal GAG metabolism appears to be a pivotal mechanism for EC dysfunction that is associated with various vascular complications of diabetes. The “steno hypothesis”, first advanced by Deckert *et al*., in 1989, held that a genetic defect in the regulation of heparan sulfate produced by endothelial, myomedial, and mesangial cells determines the susceptibility of diabetic patients to develop proteinuria and angiopathy with its associated cardiovascular risk. Some diabetic patients have a genetic trait leading to lower activity of the enzymes response for GAG sulfation under hyperglycemic conditions (Gambaro and van der Woude, 2000). Studies using biochemical techniques to measure GAG content of kidneys, obtained at autopsy, demonstrated that the glomerular basement membrane (GBM) of the patients with diabetic nephropathy contained less GAG than the

kidneys of nondiabetic control subjects (Parthasarathy and Spiro, 1982; Shimomura and Spiro, 1987). Similar changes in HS content in the intima of the aortas of patients with diabetes mellitus have been observed (Wasty *et al*., 1993), suggesting that the abnormalities in HS metabolism are not necessarily restricted to the kidney. This would explain the association between diabetic cardiovascular mortality and nephropathy. Using HSPG monoclonal antibody (JM-403), some studies showed that decreased GBM staining intensity correlated with the degree of proteinuria in diabetic nephropathy (Tamsma *et al*., 1994) and staining of skin basement membrane was also significantly reduced in patients with diabetic nephropathy compared to control diabetic patients with long-standing diabetes without nephropathy (van der Pijl *et al*., 1998). Other studies also showed decreased serum HS and increased urine GAGs in patient with overt diabetic nephropathy (Yokoyama *et al*., 1999; Perez-Blanco *et al*., 2000). Subcutaneous exogenous heparin significantly reduced proteinuria in patient with diabetic nephropathy (Myrup *et al*., 1995).

1.3.5. Basic Fibroblast Growth Factor (bFGF) Released by HSPG Degradation

The function of growth factors is to couple cells to their environment and provide the cells with plasticity to respond appropriately to external changes or to changes in their own state (Sporn and Roberts, 1993). Binding of growth factors to cell receptors, can transduce a signal to the cell nucleus.

The fibroblast growth factors (FGFs) are a family of cytokines, a group of polypeptides characterized by high affinity to heparin and HSPG which affect the growth, differentiation, and migration of many cell types (Folkman and Klagsbrun, 1987). The affinity of bFGFs for HSPGs limits bFGF diffusion and release into the interstitial space (Moscatelli, 1987; Flaumenhaft *et al*., 1990). bFGF molecules probably remain mostly intracellular (Vlodavsky *et al*., 1987), but are normally stored in the vascular basement membranes (Baird and Ling, 1987) where PGs serve as a reservoir for growth factors. When needed for wound healing, including vascular cells damaged by hyperglycemia (Folkman and Klagsbrun, 1987), bFGF is released by endothelial damage (Muthukrishnan *et al*., 1991) and mobilized by enzymatic hydrolysis from the vascular basement membrane (Baird and Ling, 1987). bFGF has high potential for proliferation of capillary endothelial cells *in vitro* and only picomolar quantities can promote angiogenesis *in vivo* (Broadley *et al*., 1989; Folkman and Klagsbrun, 1987). The biological effects of FGFs are exerted through interaction with the FGF receptor (FGFR). The FGFRs are a family of transmembrane tyrosine kinases which are activated upon ligand binding (Zimmer *et al*., 1993). Binding of bFGFs to bFGFRs results in autophosphorylation of receptors and signaling to the cell (Bellot *et al*., 1991). Heparin/HS is a constituent of the FGF/FGFR signaling complex and can modulate FGF activity and specificity (Ornitz, 2000). Some enzymes that synthesize and degrade HS also affect the function of FGFs. Many studies have shown that bFGF can be released from HS in the ECM by enzymes

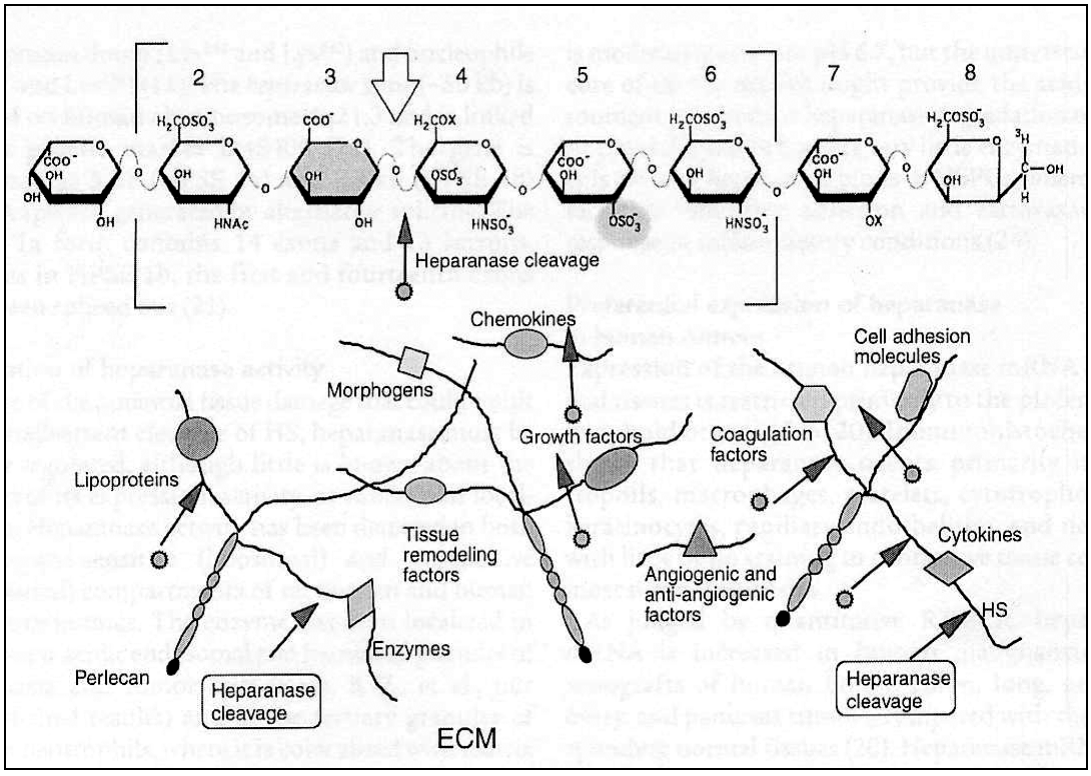
such as heparanase, trypsin or plasmin (Bashkin *et al*., 1990; Saksela *et al*., 1990). Inhibition of heparanase activity can inhibit bFGF release (Ishai-Michaeli *et al*., 1990). Heparanases break down HS, remove HS from its protein core and also degrade HS into oligosaccharides (Bame, 2001). As well, plasmin digests the protein core of HSPG (Giardino *et al*., 1994) and reduces HS production in vascular cells (Kaji *et al*., 1994). Both heparanases and plasmin interfere with binding of HSPG to bFGF/bFGFR forming a bFGF/HS /bFGFR complex, and therefore, decrease the stability of bFGF and result in loss of bFGF due to lack of HS. This lack of HS can be corrected by exogenous heparin (Yayon *et al*., 1991; Nissen *et al*., 1999). A study of nonenzymatic glycosylation of bFGF showed that the decreased activity of bFGF in hyperglycemia (Giardino *et al*., 1994) could be prevented by heparin/HS (Nissen *et al*., 1999).

1.4. Heparanase

1.4.1. The Properties of Heparanase

Heparanase is an endo-β-D-glucuronidase capable of cleaving glycosidic bonds of heparan sulfate side chains at a limited number of sites with a hydrolase mechanism. The degraded HS fragments are still an appreciable size (5-7 KDa) (Vlodavsky and Friedmann, 2001; Freeman and Parish, 1998). This suggests substrate specificity of the enzyme recognizing a particular and relatively rare HS structure (Bourin and Lindahl, 1993; Camejo *et al*., 1998). Particularly, a 2-O sulfate group on a hexuronic acid

residue located two monosaccharide units away from the cleavage site appears to be essential for substrate recognition by heparanase (Conrad, 1998) as shown in Figure 1.5. Among the cell types, including platelets, cytotrophoblasts, mast cells, neutrophils, macrophages, and T and B lymphocytes, that express heparanase under normal physiological conditions, platelets have high levels of heparanase (Freeman and Parish, 1998; Freeman *et al*., 1999). In fact, serum heparanase is mainly derived from activated platelets (Ihrcke *et al*., 1998). The content of heparanase in the kidney is second to the placenta among various organs. Furthermore, heparanase is only rarely expressed either in human ECs or in bovine aortic ECs exposed to various physiological activators (Godder *et al*., 1991; Nakajima *et al*., 1986). ECs are able to bind, internalize, and degrade high-M_r heparin, and since HS is similar to heparin, this suggests that ECs contain an endoglucosidase that degrades HSPG intracellularly (Barzu *et al*., 1987; Godder *et al*., 1991).

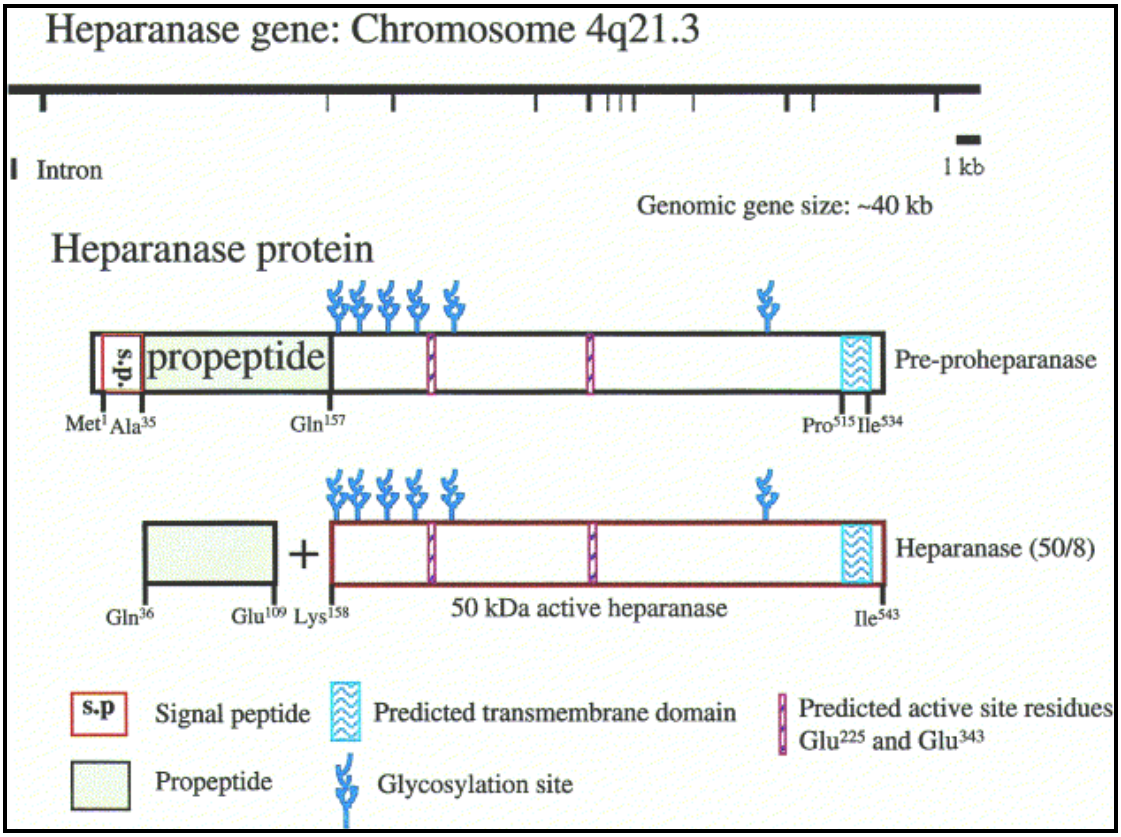


(Vlodavsky and Friedmann, 2001)

Figure 1.5. Cleavage of HSPG by Heparanase

Heparanase cleaves HSPGs (arrows) and releases a variety of physiologically and pathologically important molecules. Inset shows the heparanase recognition and cleavage site. X in sugar unit 4 represents hydrogen or SO_3^-.

Recently, partial sequencing of heparanase purified from human placenta, platelets, and hepatoma cells led to the cloning of a cDNA and gene encoding the heparanase protein (Vlodavsky *et al*., 1999; Hulett *et al*., 1999; Toyoshima and Nakajima, 1999). Heparanase gene is located on human chromosome 4q21.3. The heparanase cDNA contains an open reading frame of 1629 bp encoding a 61.2 KDa polypeptide of 543 amino acids. The mature active 50 KDa enzyme has its N-terminus 157 amino acids downstream from the initiation codon (Vlodavsky *et al*., 1999; Toyoshima and Nakajima, 1999; Fairbanks *et al*., 1999) suggesting post-translational processing of the heparanase polypeptide at an unusual cleavage site as shown in Figure 1.6. Processing and activation occur during incubation of the full-length 65 KDa recombinant enzyme with several normal and transformed cells and to a less extent, with their conditioned medium (Vlodavsky *et al*., 1999). The heparanase precursor may bind to the cell surface, most likely to HS, and is then converted to its highly active 50 KDa form in a process accompanied by endocytosis of the processed form. This is demonstrated by the observation that heparanase activity is readily obtained after transfection of mammalian cells with cDNA encoding the entire heparanase precursor (Vlodavsky *et al*., 1999; Dempsey *et al*., 2000a).



(Vlodavsky *et al*., 2001)

Figure 1.6. Scheme of the Human Heparanase Gene and Protein.

Heparanase is also localized to tertiary granules of neutrophils and mast cells and is released upon tumor necrosis factor (TNF-α) and calcium ionophore treatment (Matzner *et al*., 1985; Mollinedo *et al*., 1997; Bashkin *et al*., 1990). Heparanase release by degranulation has been implicated in diapedesis and extravasation of a number of immune cells, including neutrophils, macrophages, and lymphocytes (Vlodavsky *et al*., 1992; Bartlett *et al*., 1995; Parish *et al*., 1998), while heparanase inhibitors exhibited anti-inflammatory activity (Parish *et al*., 1998). Cleavage of HS side chains by degranulated heparanase during inflammation may facilitate the passage of blood borne normal and malignant cells into tissues by altering the composition and structural integrity of the subendothelial ECM (Vlodavsky and Friedmann, 2001; Vlodavsky *et al*., 1992**;** Bartlett *et al*., 1995). Heparanase may facilitate the release of a multitude of HS bound growth factors, cytokines and chemokines that would enhance the immune reaction or activate the vascular endothelium (Vaday and Lider, 2000; Elkin *et al*., 2001).

1.4.2. Pathogenesis Caused by Heparanase

HSPGs play a key role in the self-assembly and integrity of the multi-molecular architecture of basement membrane and ECMs, hence, their cleavage is likely to alter the integrity and functional state of tissue and to provide a mechanism by which cells can respond rapidly to changes in the extracellular environment. Enzymatic degradation of HS is, therefore,

involved in diverse fundamental biological and pathological phenomena associated with cell migration, including embryonic morphogenesis, angiogenesis, inflammation, neurite outgrowth, tissue repair and cancer metastasis (Wight *et al*., 1992; Bandtlow and Zimmermann, 2000; Nakajima *et al*., 1988; Vlodavsky *et al*., 1992; Parish *et al*., 1999). Since HSPGs are important constituents of blood vessels and the basement membrane, cleavage of HS is expected to facilitate extravasation of blood-borne tumor cells, as well as sprouting of angiogenic ECs by modulating growth factor activity and bioavailability. Cancer invasion and metastasis involves degradation of ECM components, including collagens, laminins, fibronectin and HSPGs. The malignant cell is able to accomplish this task through the concerted sequential action of enzymes such as matrix metalloproteinases (MMPs), serine and cysteine proteases, and endoglucosidases. Expression of heparanase is associated with the metastatic potential of tumor cells (Vlodavsky *et al*., 1999; Hulett *et al*., 1999; Parish *et al*., 2001; Nakajima *et al*., 1988; Vlodavsky *et al*., 1994) and increased levels of heparanase have been found in sera of animals and human cancer patients bearing metastatic tumors (Nakajima *et al*., 1988).

1.4.3. Heparanase in Diabetes

In the patient with overt diabetic nephropathy, HS and HSPG were found to be decreased by using newly developed monoclonal antibodies with reactivity to human HS and HSPG core proteins (Tamsma *et al*., 1994; van

den Born *et al*., 1992; 1993). A decreased intensity of HS staining correlated with proteinuria expressed as a function of creatinine clearance (Tamsma *et al*., 1994) suggesting alteration of HSPG side chains relevant in the pathogenesis of diabetic nephropathy. The abnormalities in HSPG metabolism were also observed in the intima of the aortas of diabetic patients suggesting changes in HSPG could occur in both the macro- and micro-vasculature. Heparanase cleaves HS at specific interchain sites and induces loss of the anionic barrier on the luminal surface of endothelium resulting in a widespread rise in vascular permeability and vasculopathy. This result was determined by observing an increased GBM permeability after perfusion with bacterial heparinase (Rosenzweig and Kanwar, 1982).

Heparanase activity was found in the urine of some diabetic patients associated with poor glycemic control (Katz *et al*., 2002). Although heparanase protein was located in both the glomerular mesangial and epithelial cell lysates, there was little or no detectable activity in intact cells and their conditioned medium (Katz *et al*., 2002). This result was confirmed by immunohistochemical staining showing the presence of the heparanase protein in both the glomeruli and the tubules of normal human and rat kidney (Levidiotis *et al*., 2001).

1.5. Summary

There is evidence from many studies that endothelial dysfunction is the predisposing factor that causes diabetic vascular complications due to its

unique location and physiological properties in the vasculature. Deckert *et al*., established the “steno hypothesis” in 1989 which indicated that the genetic dysregulation of HS/GAG production was associated with diabetic cardiovascular complications. Based on their hypothesis, HSPG degradation has received the greatest focus among the several mechanisms of endothelial dysfunction in diabetic studies. Evidence from studies of both humans and animals demonstrated that the content of HS and HSPG were reduced in glomerular and skin basement membrane and in the intima of aortas in diabetic patients. HSPG synthesis was also reduced in organs of diabetic rats. However, what causes HSPG degradation in the diabetic condition is still unknown. Heparanase is believed to break down HS resulting in the release of bFGF, enzyme, and plasma proteins (Vlodavsky *et al*., 2001). bFGF then is easily degraded by proteases and loses its mitogen activity (Sommer *et al*., 1989). Degradation of HSPG causes increased vascular permeability and damage to the vascular wall due to loss of negative charges and detachment of cells from basement membrane.

Recently, most studies on heparanase expression are in the field of tumor progression and metastasis. The heparanase mRNA and protein are preferentially expressed in metastatic cell lines and specimens of human breast, colon and liver carcinomas (Vlodavsky *et al*., 1999). The study of heparanase expressed by vascular ECs in vitro and angiogenic blood vessels showed that the heparanase protein is only expressed in capillaries and small sprouting blood vessels, but not in the endothelium of mature

quiescent vessels of the human tumor (Elkin *et al*., 2001**).** In addition, intact original human ECs and their lysates rarely express heparanase and exposure of ECs to various physiological agents reveal no detectable induction of heparanase release. These results suggest that ECs release heparanase only when cells are injured or during cell death (Godder *et al*., 1991). Heparanase may be synthesized in the endothelial cells under harmful stimuli such as hyperglycemia, AGEs, and inflammatory cytokines causing a reduction in the anionic sites in GBM and vessels, resulting in proteinuria or vascular disease.

The studies of heparanase expression in diabetes have been restricted to diabetic nephropathy.These studies localized heparanase protein both in glomeruli capillaries and tubular epithelium and detected heparanase activity in the urine of diabetic patients (Katz *et al*., 2002). There has been no study of heparanase levels in other diabetic cardiovascular complications except for nephropathy or in vascular endothelium injured by hyperglycemia. Therefore, we hypothesize that hyperglycemia induces endothelial injury due to the upregulation of heparanase. HSPG degradation caused by increased heparanase activity provides a possible mechanism involved in the EC injury. We also hypothesize that heparin and/or insulin and/or bFGF inhibit heparanase expression thereby protecting cells from hyperglycemic injury at cellular and molecular levels.

2. OBJECTIVES

The purpose of this study is to determine the effect of medium mimicking hyperglycemia (high concentration of D-glucose) on cultured porcine aortic endothelial cells (PAECs). Effects of high glucose or heparanase on endothelial cell injury will be determined. The ability of insulin and/or heparin and/or bFGF to protect ECs from high glucose and heparanase injury will also be determined.

The specific objectives of this study are:

- To maintain viable PAECs as an *in vitro* model of human diabetic cardiovascular complications
- To determine if PAECs are injured by high glucose (30 mM) and if this injury is prevented by heparin and/or insulin and/or bFGF
- To determine if heparanase mRNA is expressed in high glucose treated PAECs and if this expression is inhibited by heparin and/or insulin or by bFGF
- To determine if heparanase activity is expressed in high glucose treated PAECs and if this activity is prevented by heparin and/or insulin
- To determine if free radicals (H_2O_2) induce heparanase production

- To determine if an increase in osmolarity (30 mM mannitol) causes heparanase production.
- To determine if heparanase causes PAEC injury and if this injury is prevented by heparin and/or insulin and/or bFGF

3. Protection of Porcine Aortic Endothelial Cell Injury from High Glucose by Insulin, Heparin and bFGF

3.1. Introduction

Diabetes mellitus (DM) is a group of diseases characterized by hyperglycemia and associated with vascular complications including microangiopathy and macroangiopathy (Ruderman *et al*., 1992; Lee *et al*., 1989a). The hallmarks of microangiopathy are diabetic retinopathy and nephropathy leading to blindness and renal failure (Ruderrman *et al*., 1992; Kamata *et al*., 1992; Aiello *et al*., 1994; Pirart, 1977). Macroangiopathy in diabetes, includes coronary artery disease, peripheral vascular disease, cerebrovascular disease, and is the result of an acceleration of atherosclerosis and increased thrombosis, thus increasing the risk of myocardial infarction, stroke and ischaemia (Mogensen *et al*., 1988; Jarrett *et al*., 1982; Mykkanen *et al*., 1990).

The endothelium, a simple monolayer of metabolically active cells lining the inner surface of all blood and lymphatic vessels, plays an important role in the structural integrity and normal function of the circulatory system. The endothelium synthesizes and releases many vasoactive substances including endothelial derived nitric oxide (NO), prostaglandin I_2 ($PGI_{2)}$, tissue plasminogen activator (tPA), plasminogen activator inhibitor-1 (PAI-1),

angiotensin II, angiotension-converting enzyme (ACE), endothelin-1 (ET-1), transforming growth factor- *beta* (TGF-*β*), etc. to maintain endothelial integrity, regulate the vascular tone and to maintain blood fluidity and homeostasis (Webb and Cockcroft, 1990; Cohen and Tesfamariam, 1992; Stehouwer, 1998).

Since the initial injury in diabetes mellitus takes place in the blood vessel, the endothelial cells (ECs) are considered to be the first cells targeted by hyperglycemia. Under hyperglycemic conditions, the normal function of ECs is perturbed due to loss of its antithrombotic and profibrinolytic nature, increase in platelet adhesion and aggregation, and decrease in NO mediated vasodilation (Stehouwer, 1998; Elliott *et al*., 1993). A considerable body of evidence in human studies indicates that endothelial dysfunction is closely associated with the development of retinopathy, nephropathy and atherosclerosis in diabetes mellitus (Stehouwer and Schaper, 1996; Stehouwer *et al*., 1997).

Hyperglycemia is almost certainly the predisposing factor in the development of diabetic complications. Patients under better glycemic control developed fewer eye and/or renal complications (Pirart, 1977). Hyperglycemia causes endothelial dysfunction through several mechanisms including the accumulation of sorbitol, alterations in the cellular redox state, changes in the regulation of protein kinase C (PKC), decrease in Na/K-ATPase and formation of nonenzymatic glycosylation of proteins (Tilton *et al*., 1989; Pugliese *et al*., 1990; Bank *et al*., 1989; Greene *et al*., 1987; Lee *et*

al., 1989a; Craven and DeRubertis, 1989b; Williamson *et al*., 1990; Tsilibaru *et al*., 1988; Charonis *et al*., 1990; Sensi *et al*., 1989). Hyperglycemia also causes cell damage contributing to cell loss and progressive microvascular occlusions in the eye and kidney, as well as intimal proliferation and occlusion in the macrovasculature (Kohner *et al*., 1982; Bresnick *et al*., 1997; Steffes *et al*., 1989; Osterby, 1990).

Although there are two broad categories of ECs, namely macro (large vessel) ECs and micro (small vessel) ECs, based on their site of origin with their own different properties, both express the pathological features associated with diabetic complications. These features are exaggerated proliferation of ECs and thickening of the basement membrane (BM) (Fischer *et al*., 1979; Kefalides, 1981; Osterby, 1990). Endothelial oxidative stress represents another common pathological mechanism for atherosclerosis in diabetes (Ceriello *et al*., 1996).

Among the pathogenesis of diabetic endothelial dysfunction, depletion of heparan sulfate (HS) and/or abnormal glycosaminoglycan (GAG) metabolism appear to be a pivotal mechanism that is associated with various vascular complications. Heparan sulfate proteoglycan (HSPG) and HS were decreased in glomerular basement membrane (GBM) in patients with overt diabetic complications which correlated with the degree of proteinuria (Tamsma *et al*., 1994; van den Born *et al*., 1992; 1993). Similar changes in HS content in the intima of aortas of diabetic patients have been observed (Wasty *et al*., 1993). The skin basement membrane in patients with diabetic

nephropathy was also significantly reduced compared to diabetic patients without nephropathy and a reduced ^{35}S-labeled HSPG synthesis was observed in aorta, liver and intestinal epithelium of experimentally diabetic rats (Brown *et al*., 1982; Kjellen *et al*., 1983; Levy *et al*., 1984). This evidence demonstrates that changes in HS metabolism could occur in any tissue or organ in addition to the kidney in the diabetic condition, suggesting the link between HS abnormalities and vascular complications both in large and small vessels in diabetes.

HSPGs are synthesized by vascular ECs and incorporated into the cell surface and subendothelial basement membrane and extracellular matrix (ECM) (Muir, 1997; Simionescu and Simionescu, 1993; Hook, 1984). HSPGs with their negative charged sulfate and carboxylate residues, create a “charge barrier”, thought to play an important role in controlling vascular permeability to the anionic plasma proteins (Hardebo and Kahlstrom, 1985). HSPGs interacting with fibronectin, laminin and collagen in ECM are responsible for maintaining the integrity of the vessel wall (Kraemer, 1971; Hedman *et al*., 1982). HSPGs act as a reservoir for HS related growth factors such as bFGF. Binding of bFGF to HS ensures the normal function of bFGF for cell growth and proliferation. Degradation of HSPGs could cause ECs to lose their negative charge and disassemble from the ECM leading to an increase in vascular permeability, damage to the vessel wall, and changes in growth factor activity.

Heparanase is an endo-β-D-glucuronidase that cleaves HS at specific interchain sites. Under normal physiological conditions, heparanase is expressed in platelets, cytotrophoblasts, mast cells, neutrophils, macrophages, and placenta (Freeman and Parish, 1998; Freeman *et al*., 1999). Degradation of HS by heparanase results in the release of growth factors, enzymes and plasma proteins (Vlodavsky and Friedmann, 2001). Under pathological conditions, heparanase activity is found in cancer cells with metastatic potential and in activated cells of the immune system (Parish *et al*., 2001; Vlodavsky *et al*., 1999). Heparanase activity was also found in the urine of some diabetic patients and heparanase protein was expressed in both the glomerular mesangial and epithelial cell lysates, but not in intact cells (Katz *et al*., 2002). It is possible that EC injury by hyperglycemia is due to HSPG degradation by upregulation of heparanase. Thus we wished to determine if heparanase was upregulated in high glucose treated ECs.

Insulin, heparin and a combination of insulin and heparin prevented the intercellular gaps of ECs cultured in high glucose (Mandal *et al*., 2000). Evidence from several previous studies has shown that insulin has the ability to increase production of NO in cultured ECs to ensure the normal function of blood vessels (Zeng and Quon, 1996; Vallance *et al*., 1989). As well, heparin can accumulate in ECs at a greater concentration than in plasma, can increase amounts of HS on the EC surface, and can prevent ECs from free radical injury (Hiebert *et al*., 1993; Nader *et al*., 1991; Hiebert and Liu, 1990). Therefore, we postulated that insulin and/or heparin would protect ECs from

high glucose injury synergistically through protecting HSPG from degradation by inhibiting heparanase production.

bFGF has a high affinity for heparin and HSPGs which is required for bFGF activity but also limits bFGF diffusion and release into interstitial spaces (Moscatelli, 1987; Flaumenhafl *et al*., 1990). The high affinity of bFGF for heparin and HSPG together with the high potential of bFGF for proliferation in cultured capillary ECs (Broadley *et al*., 1989) may prevent HSPG degradation. Previous studies have shown that both heparanase and plasmin decreased the stability of bFGF/HS/bFGFR complex resulting in loss of bFGF due to HS degradation which was corrected by exogenous heparin (Yayon *et al*., 1991; Nissen *et al*., 1999). As well the decreased activity of bFGF in hyperglycemia due to nonezymatic glycosylation was prevented by heparin/HS (Giardino *et al*., 1994; Nissen *et al*., 1999). Thus, the stabilization of bFGF/HS/bFGFR complex, by supplying heparin and bFGF, may protect ECs from injury by high glucose or heparanase.

The purpose of our study was to determine if hyperglycemic concentrations of D-glucose induce cultured EC injury and if heparanase had a similar damaging effect on ECs. As well, we wished to determine if supplementation of insulin and/or heparin and/or bFGF could protect cells from high glucose or heparanase injury demonstrating their protective effect on HSPG degradation of ECs.

3.2. Methods and Materials

Isolation of Porcine Aortic Endothelial Cells (PAECs)

Porcine aortic endothelial cells (PAECs) were cultured according to the method of Gotlieb and Spector (1981). Primary cultures of PAECs were obtained directly from the porcine aorta. The aorta segment was cut and suspended in calcium- and magnesium-free Dulbecco's phosphate-buffered saline (CMF-DPBS) three times, while connective tissue was trimmed away from the outside of the aorta and small pieces were cut off of each end. One end of the aorta was clamped with two hemostats (ensuring no leakage from bottom or branch points). The lumen of the aorta was rinsed with DPBS three times and then filled with collagenase solution (Type IV, Sigma, St. Louis, MO, USA; 1 mg/ml in CMF-DPBS at 37˚C). Collagenase solution was removed after approximately 6 minutes and the endothelial surface of the aorta was gently rinsed with M199 (GibcoBRL, Life Technologies, Inc., Grand Island, NY, USA) containing 5% fetal bovine serum (FBS, GibcoBRL, Life Technologies, Inc.,), 50 μg/ml penicillin and 10 μg/ml streptomycin. The medium in the aorta was removed and plated onto 60 mm tissue culture dishes. The volume was made up to 2 ml medium following plating. Cells were incubated at 37˚C with 5% CO_2 / 95% air in a humidified environment. Endothelial cells were identified by their morphological appearance of a monolayer of cobblestone-like flattened cells and the presence of vWF in initial cultures. Non-endothelial-like cells, such as smooth muscle cells and fibroblasts were destroyed by mechanical suction before the first passage.

PAEC Culture Conditions

To subculture the cells, confluent 60 mm dishes of PAECs were washed twice with sterile CMF-DPBS, followed by exposure to a sterile 0.025% trypsin solution for two or three minutes at room temperature. To ensure all cells were suspended, the medium with cells was pipetted back and forth in the dishes. The cells were then resuspended in 6 ml of culture medium and seeded onto three 60 mm dishes (2 ml/dish). The cells become confluent in 4 to 5 days. The cells were continually subcultured, with or without a freezing step for storage, up to passage 3 and were then seeded onto 35 mm dishes (usually one 60 mm dish was passed to six 35 mm dishes) for use in experiments which become confluent in 3 to 4 days.

For freezing cells, PAECs were washed twice with CMF-DPBS, were harvested by 0.025% trypsin, and resuspended in culture medium. The cell suspension was centrifuged at 1,500 rpm for five minutes, and then the cells were resuspended in the freezing medium containing 60% of FBS, 30% culture medium and 10% dimethyl sulfoxide (DMSO) (V/V). After cooling for 1 hr at 4˚C, cells were frozen at - 70˚C. When required, frozen cells were thawed at room temperature and seeded onto 60 mm dishes with fresh culture medium. The cell medium was changed after the cells attached to the bottom of the dishes, about two hours later.

Reagents Used in PAEC Treatment

D-glucose, heparin and insulin were first prepared as a stock solution in CMF-DPBS. The concentration of stock solution for glucose (D-Glucose, BDH Inc. Toronto, Canada) was 540 mg/ml (3M), bovine lung heparin (151 USP U/mg Upjohn Pharmaceuticals, Kalamazoo, MI, USA) was 0.1 mg/ml, insulin (Humulin® N) was 100 U/ml. Heparinase I and bFGF were prepared in M199 without serum. The concentration of stock solutions for heparinase I (SIGMA) was 10 U/ml and 1 U/ml and for bFGF (SIGMA) was 0.1 ng/µl.

Addition of High Glucose, Heparin, Insulin and bFGF to PAEC Medium

Cell medium was changed just prior to addition of reagents. For addition of high glucose, 10 µl of 540 mg/ml (3 M) glucose stock solution was added to 1 ml medium to give a final concentration of 5.4 mg/ml (30 mM). For heparin addition, 5 µl of 0.1 mg/ml heparin stock solution was added to 1 ml medium to give a final concentration 0.5 µg/ml. For insulin addition, 10 µl of 100 U/ml insulin stock solution was added to 1 ml medium to give a final concentration of 1 U/ml. For bFGF addition, 10 µl of 0.1 ng/µl of bFGF was added to medium to give a final concentration of 1 ng/ml. Cell medium was changed and glucose, insulin, heparin and bFGF were added fresh every other day for seven days. Cells were harvested 48 hrs after the last addition of reagents.

Determination of Culture Conditions and Optimal Doses of Heparinase I Damage to PAECs

In order to determine the culture conditions and damaging doses of heparinase I in PAECs, three experiments were done. Concentration of heparinase I, 0.01 U/ml, 0.05 U/ml, 0.1 U/ml, 0.3 U/ml and 0.5 U/ml in medium, was produced by adding 10 µl of 1 U/ml of heparinase I and 5µl, 10 µl, 30 µl and 50 µl of 10 U/ml of heparinase I to 1 ml cell medium respectively. Cells were cultured either in medium with serum for six or ten days, where cell medium was changed and fresh heparinase I was added every other day; or in serum free medium for two days by adding heparinase I once.

Addition of Heparinase I, Heparin, Insulin and bFGF to PAEC Medium

Cell medium was changed. For heparinase I addition, 30 µl of 10 U/ml of heparinase I was added to 1 ml cell medium first to give the final concentration of 0.3 U/ml. Then heparin, insulin or bFGF was added to medium as described above. Fresh reagents were added once. Cells were harvested after culture in medium without serum for two days.

Assessment of Cell Injury

Trypan blue exclusion: Number of viable cells was determined by using trypan blue dye. Trypan blue is an exclusion dye for distinguishing

viable cells in suspension. Live cells excluding dye are not stained blue, while dead cells incorporating the dye are stained blue. To assess the extent of cell injury, cell medium was removed and 100 µl was saved in a microcentrifuge tube for LDH determination. The cells were washed with CMF-DPBS, and then detached using 0.5 ml of 0.025% trypsin for 2-3 minutes and then cells were suspended in I ml of culture medium. An aliquot of 100 µl of the cell suspension was mixed with 10 µl of 0.4% of trypan blue solution (SIGMA) for 2-3 minutes. The stained cell suspension was counted in a hemocytometer chamber with the light microscope. The number of live cells and dead cells (those taking up trypan blue) were counted and calculated in the dish. The number of live cells in experimental cultures was expressed as percent of cells in control cultures for each experiment.

Lactate dehydrogenase (LDH) assay: LDH released from damaged cells to medium is another indicator of cell injury. The Sigma Diagnostic Kit No. 228-UV was used to measure the LDH content in the medium. A 50 µl of sample was added to 500 µl of reagent and LDH was quantified spectrophotometrically by the rate of change in absorbance at 340 nm at room temperature. The increased absorbance at 340 nm is the result of reduction of NAD to reduced NADH as LDH catalyzes the conversion of lactate to pyruvate as shown by the following reactions:

$$\text{Lactate} + \text{NAD} \xrightarrow{\text{LDH}} \text{Pyruvate} + \text{NADH} + H^{+}$$

Therefore, the rate of NADH production is directly proportional to the LDH activity in the sample.

Statistical Analysis

All data were expressed as mean +/- standard error (SE) from three dishes per group. A one way ANOVA was used to determine significant differences between groups. Values of $P<0.05$ were considered to be statistically significant.

3. 3. Results

Isolation of Porcine Aortic Endothelial Cells

The protocol to isolate PAECs developed according to Gotlieb and Spector (1981) was successful and easy to perform. Although cells were contaminated by some unwanted cells, such as fibroblasts and SMVCs, these cells were easily distinguished by their morphological appearance from the cobblestone-like endothelial cells in the primary culture. These cells were then removed by mechanical suction when cells were passed the first time. Purified endothelial cells were obtained following several passages.

PAECs Injured by High Glucose were Protected by a Combination of Heparin and Insulin

In order to determine if high glucose induces PAEC injury and if injury is prevented by a combination of heparin and insulin, PAECs passage 4 were

exposed to high glucose (30 mM), insulin (1 U/ml), heparin (0.5 μg/ml) and glucose plus heparin plus insulin for seven days. Cell medium was changed and fresh reagents were added every other day. Cells were counted and LDH release in medium was determined 48 hrs after the last addition of reagents.

As shown in Figure 3.1, PAECs treated with glucose showed a significant decrease in live cell number and a significant increase in LDH release compared to control cells, suggesting that glucose injures PAECs. There were no significant changes in live cell number and LDH release in PAECs treated with heparin alone and a significantly increased live cell number in PAECs treated with insulin alone compared to control cells suggesting heparin had no effect on cells and insulin promoted cell proliferation. The combination of heparin and insulin in the presence of high glucose significantly increased live cell number and decreased LDH release compared to cells injured by high glucose alone suggesting their protective effect on cells injured by high glucose.

The Protective Effect of Insulin and/or Heparin on PAECs Injured by High Glucose

In order to determine if insulin and /or heparin protect PAECs from high glucose injury, PAECs passage 4 were treated with high glucose (30 mM), glucose plus insulin (1 U/ml), glucose plus heparin (0.5 μg/ml) and

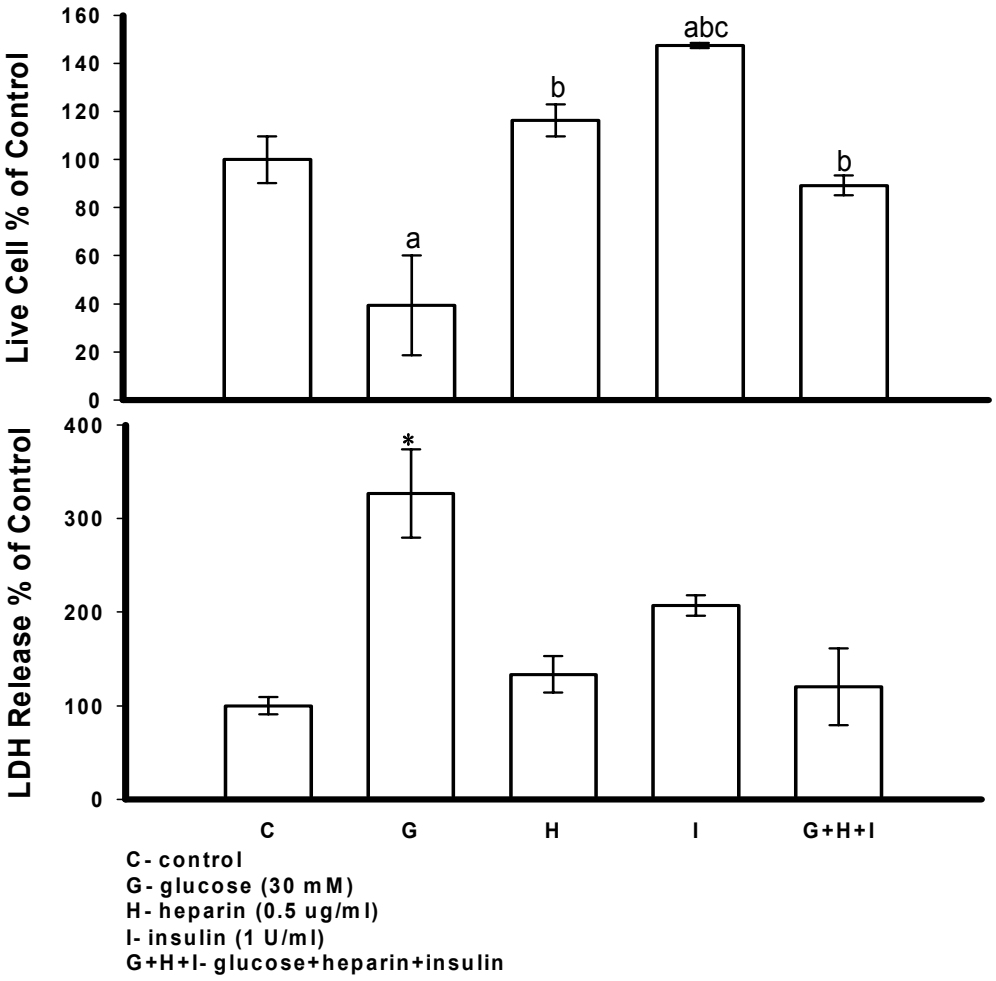


Figure 3.1. PAECs Injured by High Glucose were Protected by a Combination of Heparin and Insulin

PAECs passage 4 were exposed to glucose (30 mM), insulin (1 U/ml), heparin (0.5 μg/ml) and glucose plus heparin plus insulin for seven days. Cell medium was changed and fresh reagents were added every other day. Cells were counted and LDH release in medium was determined 48 hrs after last addition of reagents. Results are expressed as mean+/-SE of three dishes per group. For live cell number, significantly different than a, control; b, glucose; c, glucose+heparin+insulin (*P <0.001*). For LDH release, significantly different than any other group (**P<0.01*) (one way ANOVA).

glucose plus insulin plus heparin for seven days. Cell medium was changed and fresh reagents were added every other day. Cells were counted and LDH release to medium was determined 48 hrs after the last addition of reagents.

As shown in Figure 3.2, a trend towards a decrease in live cell number and a significant increase in LDH release were seen in PAECs treated with high glucose showing glucose injury to cells. As well, a significant increase in live cell number and decrease in LDH release was seen in PAECs treated with high glucose and a combination of insulin and heparin suggesting the protective effect of a combination of insulin and heparin on glucose injured cells, similar to results shown in Figure 3.1. There was a significant increase in live cell number and a corresponding significant decrease in LDH release when insulin was added to high glucose injured cells suggesting the protective effect of insulin on glucose injured cells. PAECs treated with glucose plus heparin showed no significant increase in live cell number, but a significant decrease in LDH release compared to high glucose alone.

Insulin and/or Heparin Protected PAECs from Glucose Injury When bFGF is Present in Cell Medium

In order to determine if insulin and/or heparin protected PAECs from glucose injury when bFGF is present in cell medium, PAECs passage 4 were treated with high glucose (30 mM), glucose plus bFGF (1 ng/ml), glucose plus bFGF plus insulin (1U/ml), glucose plus bFGF plus heparin (0.5 µg/ml) and

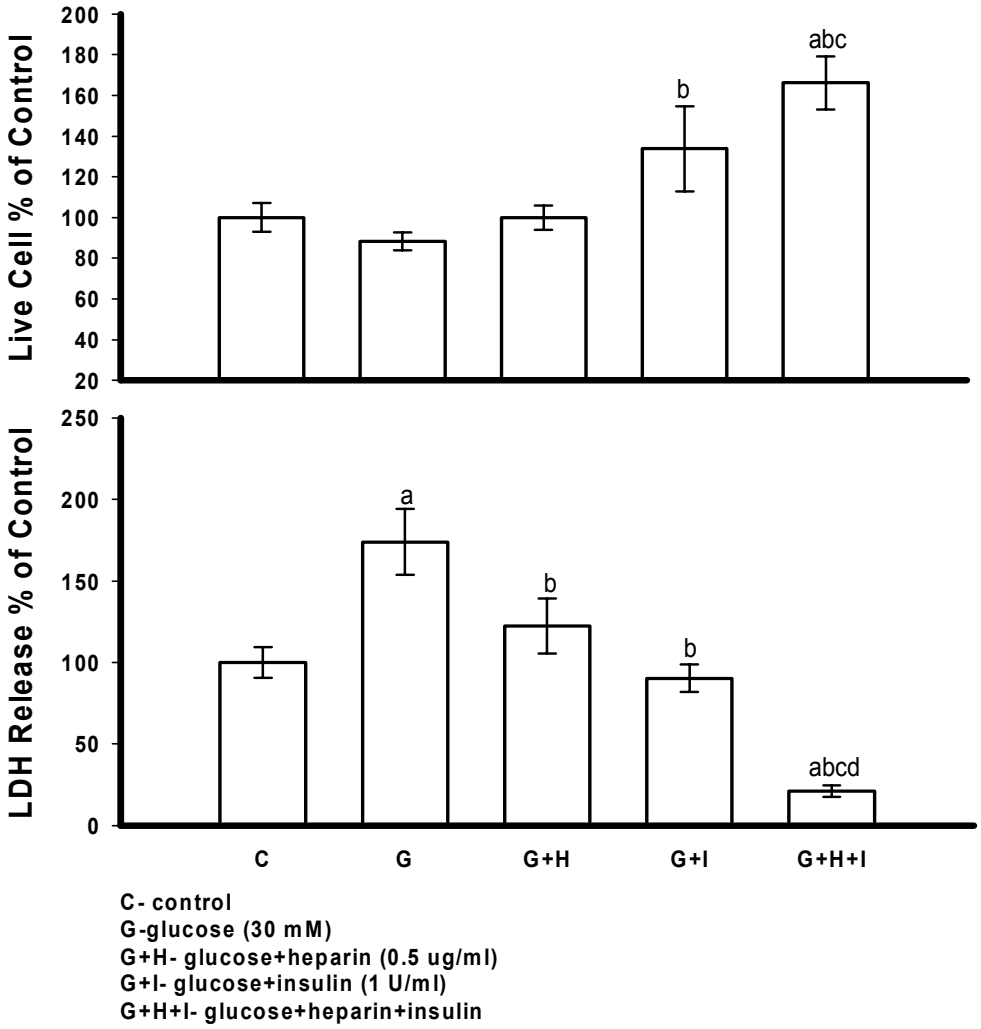


Figure 3.2. The Protective Effect of Insulin and/or Heparin on PAECs Injured by High Glucose

PAECs passage 4 were treated with glucose (30 mM), glucose plus insulin (1 U/ml), glucose plus heparin (0.5 μg/ml) and glucose plus insulin plus heparin for seven days. Cell medium was changed and fresh reagents were added every day. Cells were counted and LDH release to medium was determined 48 hrs after the last addition of reagents. Results are expressed as mean+/-SE of three dishes per group. For live cell % of control, significantly different than a, control; b, glucose; c, glucose + heparin ($P<0.01$). For LDH release, significantly different than a, control; b, glucose; c, glucose+heparin; d, glucose+ insulin ($P<0.001$) (one way ANOVA).

glucose plus bFGF plus insulin plus heparin for seven days. Cell medium was changed and fresh reagents were added every other day. Cells were counted and medium LDH was determined 48 hrs after the last addition of reagents.

As shown in Figure 3.3, a significant decrease in live cell number and increase in LDH release was shown in high glucose treated cells versus control cells showing high glucose damages cells. The combination of insulin and heparin had a protective effect on high glucose injured cells when bFGF was present in cell medium as shown by a significant increase in live cell number and decrease in LDH release in cells treated with glucose plus bFGF plus insulin plus heparin versus high glucose alone. In addition, a significant increase in live cell number and decrease in LDH release was shown in cells treated with high glucose plus insulin plus bFGF versus glucose alone suggesting the protective effect of insulin on glucose injured cells when bFGF is present in cell medium. Heparin with bFGF and bFGF alone added to cells injured by high glucose showed a significant increase in live cell number versus high glucose treatment alone. Although LDH release was less in high glucose plus heparin plus bFGF and high glucose plus bFGF treated groups versus the high glucose alone treated group, this difference did not reach significance. The combination of insulin plus bFGF, and insulin plus heparin plus bFGF was more protective than bFGF and bFGF plus heparin on high glucose treated cultures, when numbers of live cells were

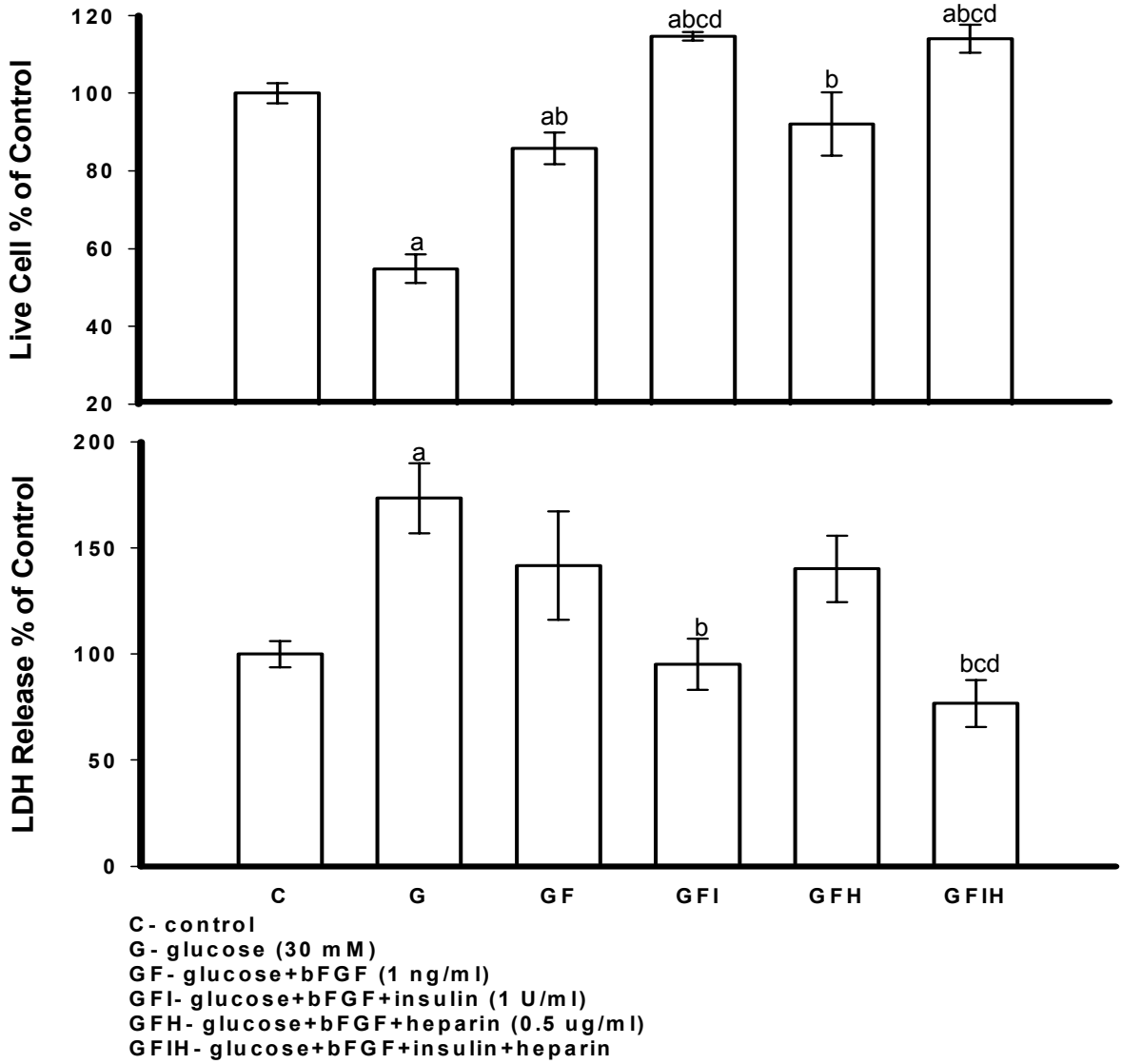


Figure 3.3. Insulin and/or Heparin Protected PAECs from High Glucose Injury when bFGF was Present in Cell Medium

Passage 4 PAECs were treated with glucose (30 mM), glucose plus bFGF (1 ng/ml), glucose plus bFGF plus insulin (1U/ml), glucose plus bFGF plus heparin (0.5ug/ml) and glucose plus bFGF plus insulin plus heparin for seven days. Cell medium was changed and fresh reagents were added every other day. Cells were counted and medium LDH was determined 48 hrs after the last addition of reagents. Results are expressed as mean+/-SE of three dishes per group. For live cell % of control, significantly different than a, control; b, glucose; c, glucose+bFGF; d, glucose+bFGF+heparin ($P<0.01$). For LDH release, a, significantly different than a, control; b, glucose; c, glucose+bFGF; d, glucose+bFGF+heparin ($P<0.05$) (one way ANOVA).

considered. The combination of insulin plus heparin plus bFGF was more protective than bFGF plus heparin when LDH was considered.

Determination of the Optimal Damaging Dose of Heparinase I

A. Heparinase I dose response in PAECs cultured in M199 with serum when treated for six days

PAECs were exposed to different doses of heparinase I (0.01 U/ml, 0.05 U/ml, 0.1 U/ml and 0.3 U/ml) for six days. Cell medium was changed and fresh heparinase I was added to cell medium every other day. Cells were counted and LDH release to medium was determined 24 hrs after the last addition of heparinase I.

As shown in Figure 3.4, there are no significant differences in live cell number and LDH release in control cultures compared to those treated with different doses of heparinase I, suggesting that heparinase I did not injure cells grown and treated in M199 with serum.

B. Heparinase I dose response in PAECs cultured in M199 with serum when treated for 10 days

PAECs were exposed to different doses of heparinase I (0.01 U/ml, 0.05 U/ml, 0.1 U/ml and 0.3 U/ml) for ten days. Cell medium was changed and fresh heparinase I was added to cell medium every other day. Cells were

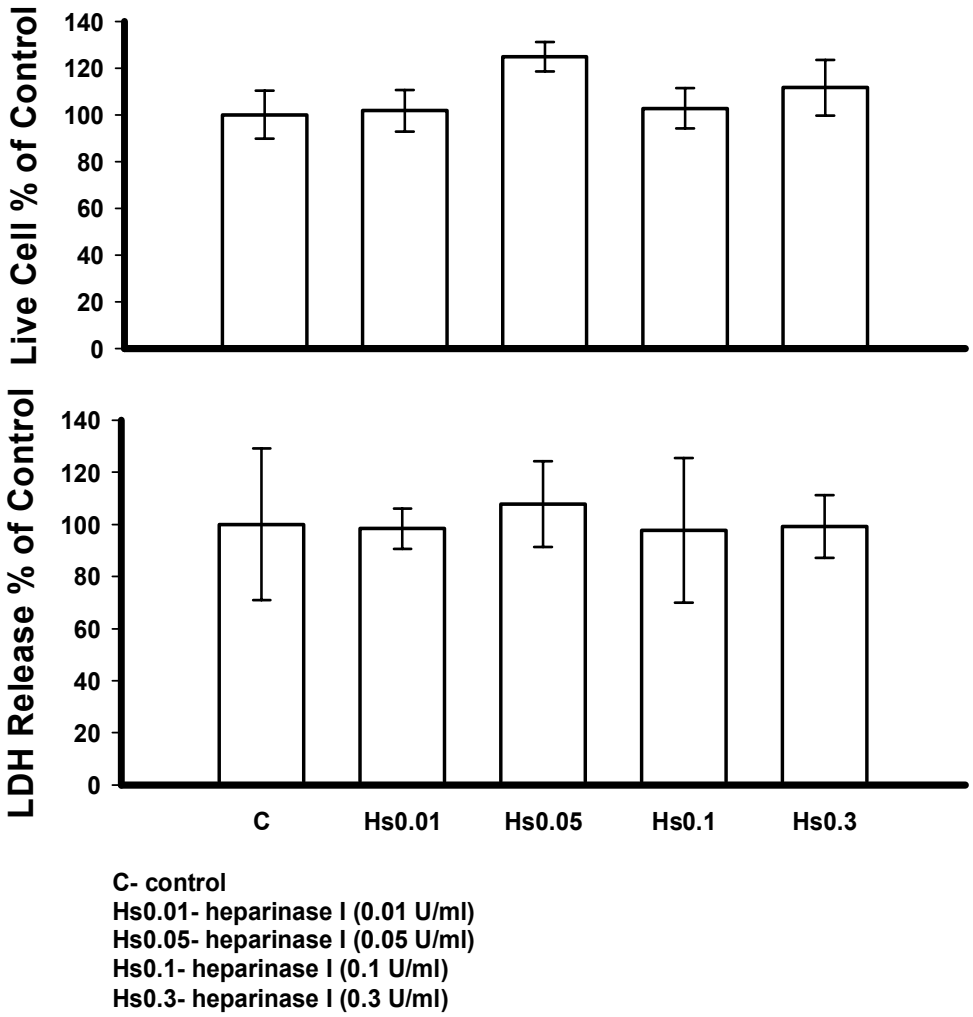


Figure 3.4. Heparinase I Dose Response Shown in PAECs Cultured in M199 with Serum when Treated for Six Days

PAECs were exposed to different doses of heparinase I (0.01 U/ml, 0.05 U/ml, 0.1 U/ml and 0.3 U/ml) for six days. Cell medium was changed and fresh heparinase I was added to cell medium every other day. Cells were counted and LDH release to medium was determined 24 hrs after the last addition of heparinase I. Results are expressed as mean+/-SE of three dishes per group(one way ANOVA).

counted and LDH release to medium was determined 24 hrs after the last addition of heparinase I.

As shown in Figure 3.5, there are no significant differences in live cell number between groups treated with different doses of heparinase I when these groups were compared to control. Although there is a significantly greater LDH release with increased doses of heparinase I (0.1 U/ml and 0.3 U/ml) compared to lower doses of heparinase I (0.05 U/ml), LDH release in control cells is significantly greater than lower doses of heparinase I (0.01 U/mand 0.05 U/ml). LDH release increase in the control group compared to some heparinase I treated groups suggests that cells were damaged with longer incubation time. Therefore, heparinase I had no damaging effect on PAECs exposed to M199 with serum even after 10 days of treatment.

C. Heparinase I dose response in PAECs cultured in serum free M199

PAECs were exposed to different doses of heparinase I (0.05 U/ml, 0.1 U/ml, 0.3 U/ml and 0.5 U/ml) for 48 hrs in serum free M199. Heparinase I was added to cell medium once. Cells were counted and LDH release to medium was determined.

As shown in Figure 3.6, PAECs treated with different doses of heparinase I showed a significant decrease in cell viability and significant increase in LDH release compared to the control group suggesting cells were

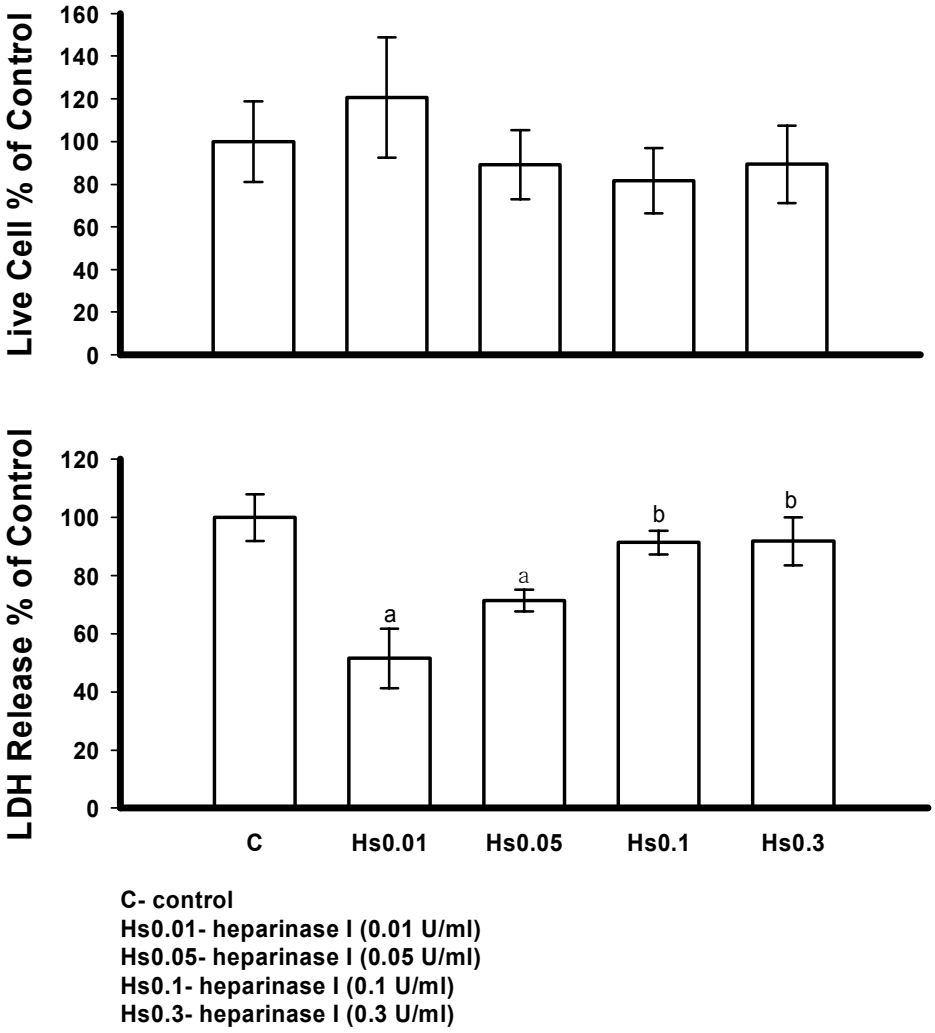


Figure 3.5. Heparinase I Dose Response Shown in PAECs Cultured in M199 with Serum when Treated for Ten Days

PAECs were exposed to different doses of heparinase I (0.01 U/ml, 0.05 U/ml, 0.1 U/ml and 0.3 U/ml) for ten days. Cell medium was changed and fresh heparinase I was added to cell medium every other day. Cells were counted and LDH release to medium was determined 24 hrs after the last addition of heparinase I. Results are expressed as mean+/-SE of three dishes per group. There were no significant changes in live cell number between groups. For LDH release, a significantly different than control; b, significantly different than heparinase I (0.01 U/ml); ($P<0.05$) (one way ANOVA).

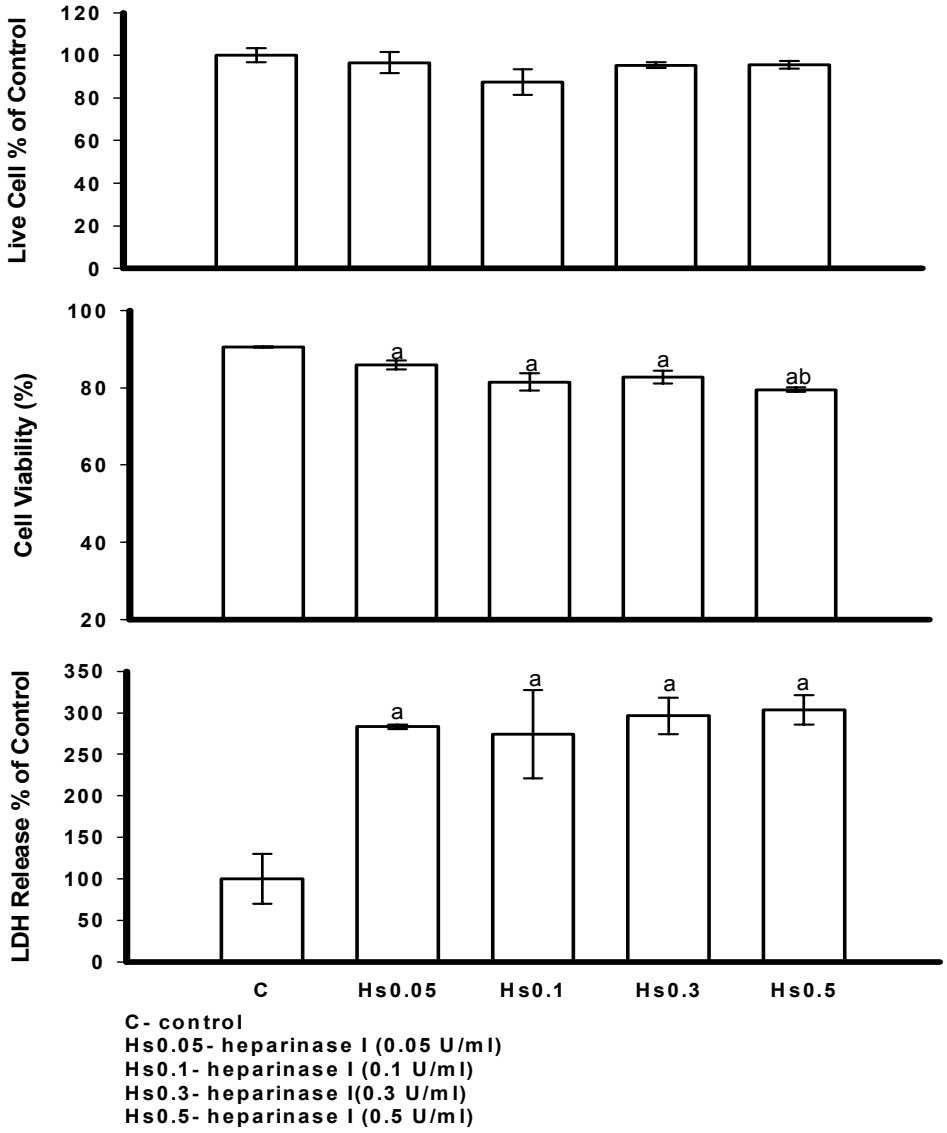


Figure 3.6. Heparinase I Dose Response Shown in PAECs Cultured in Serum Free M199

PAECs were exposed to different doses of heparinase I (0.05 U/ml, 0.1 U/ml, 0.3 U/ml and 0.5 U/ml) for 48 hrs. Heparinase I was added to cell medium once. Cells were counted and LDH release to medium was determined. Results are expressed as mean+/-SE of three dishes per group. For cell viability, significantly different than a, control; b, heparinase I (0.05 U/ml) ($P<0.05$). For LDH release, a, significantly different than control ($*P<0.05$). For live cell number, no significant difference between these groups (one way ANOVA).

injured by heparinase I when cells were exposed in serum free M199. Furthermore, there is some indication that cell injury was dose dependent since there was a significant decrease in cell viability, with heparinase I 0.5 U/ml compared to 0.05 U/ml.

Based on these three experiments we chose the doses of 0.3 U/ml heparinase I under serum free media conditions for the following experiments.

Heparinase I Induced PAEC Injury was Prevented by Insulin and/or Heparin

In order to determine heparinase I induced PAEC injury was prevented by insulin and/or heparin, PAECs passage 4 were treated with heparinase I (0.3 U/ml) and/or insulin (1 U/ml) and/or heparin (0.5 µg/ml) for 48 hrs in serum free M199. Insulin and/or heparin were added immediately after heparinase I addition. All reagents were only added once.

As shown in Figure 3.7, PAECs were injured by heparinase I as shown by a significant decrease in live cell number and increase in LDH release compared to control cells. As well insulin and heparin protected cells from heparinase I injury as shown by a significant increase in live cell number and decrease in LDH release compared to the heparinase I treated cells. Furthermore, the combination of insulin and heparin had additional protection on cells injured by heparinase I shown by a significant increase in live cell number and the lowest LDH release compared to all other groups.

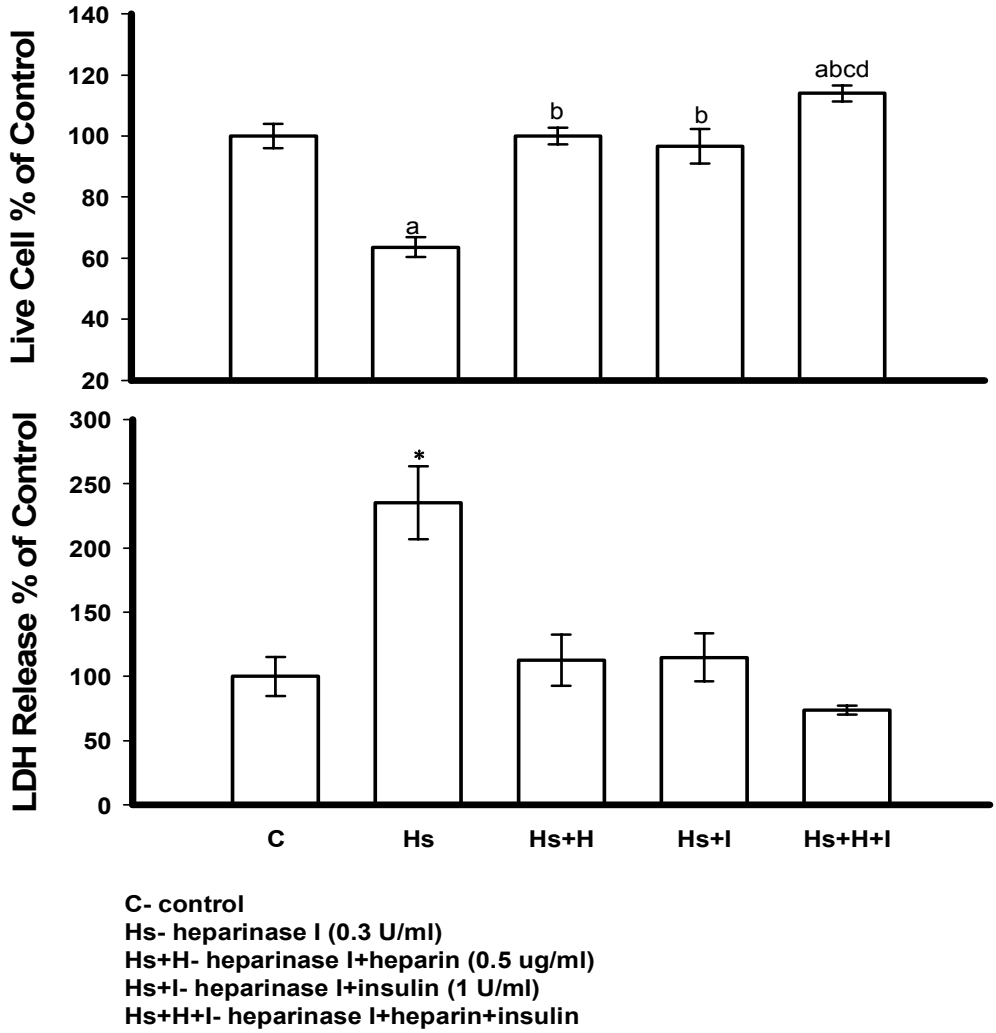


Figure 3.7. Heparinase I Induced PAECs Injury was Prevented by Insulin and/or Heparin

Passage 4 PAECs were treated with heparinase I (0.3 U/ml) and/or insulin (1 U/ml) and/or heparin (0.5 µg/ml) for 48 hrs in serum free medium 199, then cells were counted and media LDH was determined. Insulin and/or heparin were added immediately after heparinase I addition. All reagents were only added once. Results are expressed as mean+/-SE of three dishes per group. For live cell % of control, significantly different than a, control; b, heparinase I; c, heparinase I+heparin; d, heparinase I+insulin ($P<0.001$). For LDH release, heparinase group is significantly different than any other group (*$P<0.005$) (One way ANOVA).

The Protective Effect of Insulin and/or Heparin on PAECs Injured by Heparinase I When bFGF is Present in Cell Medium

In order to determine the protective effect of insulin and/or heparin on PAECs injure by heparinase I when bFGF is present in cell medium, PAECs passage 4 were treated with heparinase I (0.3 U/ml), heparinase I plus bFGF (1 ng/ml), heparinase I plus insulin (1 U/ml) plus bFGF, heparinase I plus heparin (0.5 µg/ml) plus bFGF and heparinase I plus insulin plus heparin plus bFGF for 48 hrs in serum free M199. Insulin, heparin and bFGF were added immediately after heparinase I addition.

As shown in Figure 3.8, PAECs treated with heparinase I showed a significant decrease in live cell number and increase in LDH release suggesting cells were injured by heparinase I. bFGF alone prevented LDH release in heparinase I treated cells, but had no effect on live cell number. Insulin and/or heparin and/or bFGF had a protective effect on cells injured by heparinase I shown by a significant increase in live cell number and decrease in LDH release in cultures treated with bFGF plus insulin, bFGF plus heparin and bFGF plus insulin plus heparin alone with heparinase I versus heparinase I alone. Furthermore, bFGF plus insulin plus heparin had a further protective effect on cells injured by heparinase I when live cell number but not LDH release was considered.

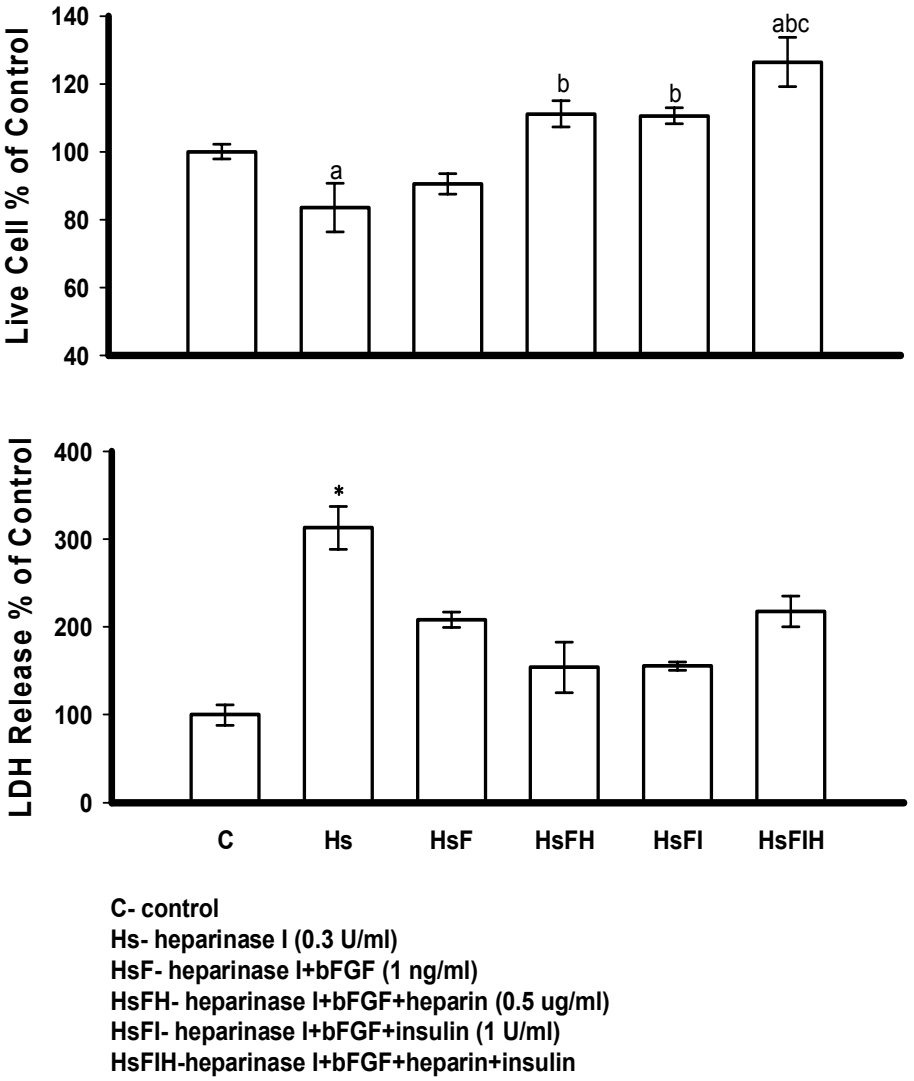


Figure 3.8. The Protective Effect of Insulin and/or Heparin on PAECs Injured by Heparinase I when bFGF was Present in Cell Medium

Passage 4 PAECs were treated with heparinase I (0.3 U/ml), heparinase I plus bFGF (1 ng/ml), heparinase I plus insulin (1 U/ml) plus bFGF, heparinase I plus heparin (0.5 ug/ml) plus bFGF and heparinase I plus insulin plus heparin plus bFGF for 48 hrs in serum free medium 199, after 48 hrs cells were counted and media LDH was determined. Insulin, heparin and bFGF were added immediately after heparinase I addition. Results are expressed as mean+/-SE of three culture dishes per group. For live cell % of control, significantly different than a, control ; b, heparinase I; c, heparinase I+bFGF ($P<0.001$). For LDH release, heparinase I treated group is significantly different than any other group ($*P<0.05$) (one way ANOVA).

3.4. Discussion

Vascular complications are the main causes of morbidity and mortality in diabetes mellitus. Endothelial cells (ECs) play many pivotal roles in the regulation of vascular tone and integrity, as well as in the maintenance of blood fluidity and homeostasis. Since the ECs are considered to be the first cells injured by hyperglycemia, endothelial damage is a contributing factor in the etiology of vascular complications of diabetes mellitus. Several mechanisms of hyperglycemia induced damage of ECs have been considered including: increased polyol pathway flux leading to redox state or increased oxidative stress, changes in the regulation of protein kinase C activated by DAG synthesis, decreased Na/K-ATPase activity and formation of nonenzymatic glycosylation of proteins. However, degradation of HSPG on the cell surface or in the ECM may play an important role in endothelial injury leading to diabetic vascular complications. Heparanase break down of HS chains in specific sites may be responsible for the degradation of HSPG contributing to endothelial injury.

Porcine aortic endothelial cells (PAECs) were used as an *in vitro* model to study human cardiovascular disease associated with endothelial injury since there is a similarity between human and porcine tissue (Lee, 1986). PAEC culture methods have been well established in our laboratory for many years. Although endothelial cells can be divided into two categories, micro and macro, based on their original site and properties, both categories present the same pathological features in diabetic complications. In this

study, using ECs from the porcine aorta (macro vessel) exposed to high glucose may represent both categories of ECs injured by hyperglycemia *in vivo*. PAECs injured by high glucose showed by decreased numbers of live cells and increased LDH release in this study could be thought of as a common indicator of EC injury associated with diabetic vascular complications. These results agree with previous observations of ECs grown under hyperglycemic conditions showing decreased proliferation and fibrinolytic potential and increased programmed cell death (Lorenzi, 1992; Baumgartner-Parzer *et al*., 1995). The present result further confirms that hyperglycemia causes cell damage and loss of cells that is the initial step leading to irreversible structural abnormalities, followed by progressive microvascular occlusion in the eye and kidney as well as intimal proliferation in large vessels (Kohner *et al*., 1982; Bresnick *et al*., 1997; Steffes *et al*., 1989; Osterby, 1990). Normal ECs can be induced to become diabetic-like cells when cultured in medium with high glucose (30 mM) for ten to twelve days (Cagliero *et al*., 1991a; 1991b), a longer period compared to the seven day treatment in our study. The diabetic-like cells however increased their proliferation significantly which was demonstrated by culturing diabetic-like ECs obtained from umbilical cords of pregnant diabetic women *in vitro* (Sank *et al*., 1994). PAECs treated with high glucose for seven days in the present study may still remain at a normal stage of cell proliferation where cell injury was shown by reduced live cell number accompanied by decreased cell proliferation. Some variation exists in the response of endothelial cells to high

glucose conditions. This variation in cell conditions, includs the use of fresh versus frozen cells, variation between animal sources, subtle differences in medium, CO_2 levels, humidity, and other unidentified factors.

Occlusion in the vasculature could also be the consequence of EC injury resulting in alterations in normal physiological properties and release of various vasoactive substances. Many studies showed that injured ECs lose their antithrombotic and antifibrinolytic nature resulting in decreased production of endothelial derived NO and increased ET-1 levels causing hypertension and narrowing of the blood vessel; and released growth factors and cytokines such as vascular endothelial cell growth factor (VEGF), bFGF and TGF-*β* promoting cell proliferation and synthesis of ECM. All the defects occurring during EC injury would result in thrombosis, atherosclerosis, tissue ischemia and ultimately vascular infarction.

Heparanase activity is involved in diverse fundamental biological and pathological processes such as embryonic morphogenesis, angiogenesis, inflammation, tissue repair and cancer metastasis. Heparanase is also expressed in the kidney and urine of diabetic patients (Katz *et al*., 2002). Since heparanase cleaves HSPG, a component of the EC surface and ECM, it may damage ECs and the vessel wall. In order to determine if heparanase damages ECs, PAECs were also treated with heparinase I alone to see if heparinase I causes EC injury similar to high glucose.

Several heparanases have been purified and characterized from platelets, placenta, and Chinese Hamster Ovary (CHO) cells named CTAP-III

(connective tissue activating peptide III), Hpa I, Hpa II and CHO cell heparanases (Karen, 2001). The commercially available heparinase I was chosen for the treatment of PAECs. Heparinase I, from *Flacobacterium heparinum (Cytophagia heparinia)*, is believed to cleave the specific glycosidic linkage in oligosaccharide substrates (Desai *et al*., 1993). Heparinase I did not cause PAEC injury when cells were cultured in M199 with serum for six to ten days, but showed a dose effect in cells cultured in serum-free medium for two days. These findings suggest that heparanase exerts its functional activity only under certain conditions. A recently discovered cell surface protein, HS/heparin-interacting protein (HIP), was involved in preventing heparanase access to its substrate HS by competing with the same binding recognition site as in the HS chain (Liu *et al*, 1997; Marchett *et al*., 1997). This could explain our observations that heparanase was active in serum free medium but not in medium with serum, because serum may contain HIP. The studies with purified Hpa I heparanase showed that at physiological pH the enzyme binds to ECM or cell surface HSPG but is inactive. Heparanase activity is optimal between pH 5.0 and 6.5, with maximal activity at pH 5.5 to 5.8, and has much less activity above pH 7.0 (Gilat *et al*., 1995; Ihrcke *et al*., 1998). It has been proposed that the pH is lowered at sites of inflammation or matrix damage which is favorable for heparanase activity (Gilat *et al*., 1995; Ihrcke *et al*., 1998; Dempsey *et al*., 2000b). Our findings showing cell injury with both high glucose and heparinase I treatment suggest that high glucose induces upregulation of

heparanase and heparanase breaks down HS causing cell injury. Metabolic effect induced by growth in 30 mM glucose may lower the medium pH (medium color become yellow). The lower pH stimulates the activation of heparanase causing HSPG degradation resulting in endothelial injury. These results further confirm that the loss of HS may play an important role in diabetic vascular complications.

Depletion and metabolic abnormalities of HS and HSPG have been found in the kidney, skin and intima of the aortas of diabetic patients with nephropathy (Tamsma *et al*., 1994; van den Born *et al*., 1992; 1993; Wasty *et al*., 1993). Exogenous heparin significantly reduced proteinuria, the first clinical finding in diabetic nephropathy (Tamsma *et al*., 1996). Heparin promotes anticoagulant, antioxidant and barrier properties of blood vessels, prevents the formation of occlusive vascular thrombi， protects against proteolytic or oxidative damage, and lowers the blood pressure (Harpel *et al*., 1996; Fairman *et al*., 1987; Peterson *et al*., 1987; Hiebert and Liu, 1990; Mandal *et al*., 1995). Based on the similarity in their basic chemical structure, heparin and HS possess common physiological, biological and pathological features in the vasculature. Previous studies showed that heparin corrected the balance between vascular collagen synthesis and degradation by acting on synthesis rather than on degradation (Caenazzo *et al*., 1997). Heparin modifies the synthesis and the structure of HSPG. The ECs possess the machinery required for the sulfation of the carbohydrate moiety of a wide array of glycoproteins (Colburn and Buonassisi, 1982; Colburn *et al*., 1987).

Therefore, supplementation of cultured ECs exposed to high glucose or heparinase I with heparin may protect cells from injury by inhibiting the degradation of HS or promoting HS synthesis. Our results showed that addition of heparin to ECs treated with heparinase I increased live cell number and decreased LDH release significantly compared to cells treated with heparinase I alone suggesting that heparin has the ability to prevent cell injury by heparanase. Even though the live cell number did not increase significantly in cells treated with high glucose and heparin compared to high glucose alone, a significant decrease in LDH release and live cell number close to control levels also indicate the potential protection of heparin on cells injured by high glucose. Taken together, these results may provide evidence of the linkage between hyperglycemia and induction of heparanase in diabetic complications. Our results are also supported by the observed reduction in albumin excretion rate after heparin treatment in diabetic patients and animals (Tamsma *et al*., 1996; Gambaro *et al*., 1992).

HS and heparin have high affinity for bFGF and play a role in the formation of the bFGF/bFGFR complex that affects the growth, differentiation and migration of many cell types (Folkman and Klagsbrun, 1989). Thus, the synthesis and degradation of HSPG is associated with bFGF function which is protected by HS synthesis and perturbed by its degradation. Our results showed a significant increase in live cell number, but not a significant decease in LDH release both in cells treated with glucose plus bFGF and glucose plus bFGF plus heparin when compared to glucose alone showing

some protective effects of bFGF and/or heparin on high glucose injured cells. However, the live cell number in control cells is significantly greater than high glucose plus bFGF indicating bFGF alone cannot protect cells from glucose injury completely. Since hyperglycemia produces many metabolic, biochemical, and functional abnormalities through several cellular pathways, the normal function of bFGF may be affected by its interaction with abnormal metabolites. Previous studies showed that in hyperglycemia, nonenzymatic glycosylation of bFGF decreased bFGF activity (Giardino *et al*., 1994) to some degree and could explain our observations here. Moreover, our present results in ECs treated with heparinase I and bFGF and/or heparin provide further evidence on the variability of the protective effect of bFGF and/or heparin on EC injury under different circumstances. Under conditions of serum free medium and without high glucose, cell injury by heparinase I is likely due only to the degradation of HSPG. The protection by bFGF of cells injured by heparinase I, showed by a significantly decreased LDH release but no change in cell number, shows the protective potential of bFGF on cells injured by heparinase I. This protective ability of bFGF is consistent with that seen in ECs treated with high glucose and bFGF. When heparin was added to ECs treated with heparinase I and bFGF, live cell number increased and LDH release decreased significantly compared to heparinase I treatment alone. This result suggests that bFGF and heparin bind together to prevent HSPG from degradation by heparinase I, which occurs without additional interacting factors normally existing in the serum or induced by high glucose.

Taken together these findings in ECs treated with heparinase I and heparin, cause us to speculate that heparin may exert its protective effect on ECs injured by heparanase in two steps: the first, heparin increases synthesis of HS on the EC; the second, the newly synthesized HS with exogenous bFGF and heparin in cell medium form the complex of bFGF/HS or heparin/ bFGFR which is the fundamental structure necessary for bFGF to play its physiological role in cell growth, differentiation, proliferation through signal transduction. These results suggest that heparin and bFGF work together to protect cells from heparanase injury.

Insulin not only stimulates cells to take up and utilize glucose to regulate blood glucose concentrations, but also promotes DNA synthesis and cell growth and replication. The latter effect was supported in this study when ECs treated with insulin alone significantly increased live cell number compared to controls. The protection by insulin of cells exposed to high glucose or heparinase I was shown in all treatment combinations in this study including insulin alone, insulin plus heparin, insulin plus bFGF and insulin plus heparin plus bFGF. The mechanism by which insulin protects ECs from high glucose injury is not entirely understood. Besides its function in regulating blood glucose and increasing cell proliferation, insulin can increase production of NO in cultured ECs (Zeng and Quon, 1996). NO is a most potent endogenous vasodilator and regulates vascular tone and blood pressure *in vivo* (Vallance *et al*., 1989) and prevents atherosclerosis by reducing oxygen consumption (Shen *et al*., 1994). Endothelial cells grown

under hyperglycemic conditions decreased their ability to produce NO due to a gene defect in regulation of nitric oxide synthase (NOS) (Chakravarthy *et al*., 1998). The combination of insulin, heparin and bFGF should exert more powerful protection on cells injured by high glucose or heparinase I. This is based on the intrinsic functions and antioxidative activity of insulin together with the multi protective effect of heparin and bFGF on injured cells discussed previously. Indeed, our present studies agreed with this speculation and showed that the combination of these three compounds was more effective than any other treatment and significantly increased live cell number and decreased LDH release compared to high glucose or heparinase I alone, heparin plus bFGF, insulin plus bFGF and bFGF alone.

In summary, our present studies demonstrate that either high glucose or heparinase I cause EC injury, but heparinase I damages ECs under limited conditions i.e. in serum free medium. It is therefore confirmed that the mechanism of EC injury by high glucose is a complicated process during which cells produce a variety of metabolic abnormalities, and the induction of heparanase may be one of them. The protective effect of heparin and bFGF alone or in combination varied depending on whether cells were exposed to high glucose or heparinase I indicating the limited damage induced by heparinase I and the complexity of glucose-induced cell injury. The cells injured by heparinase I further confirmed that the degradation of HSPG on cell surface or possibly the ECM contributes to the vasculopathy associated with EC injury that is consistent with previous observations, both *in vivo* and

in vitro, of diabetic vascular complications. Our findings are the first to show the protective effects of heparin and/or insulin and/or bFGF on cells injured by high glucose or heparinase I. However, interestingly we found that bFGF functions differently in the presence of heparin or insulin in cell medium when cells were treated with high glucose or heparinase I. The protective effect of bFGF plus heparin on ECs damaged by heparinase I is more prominent than when ECs were damaged by high glucose. However, bFGF plus insulin showed consistent protective effects in cells treated with heparinase I or high glucose suggesting that there must be a different interaction between bFGF and insulin compared to bFGF and HS or heparin. Regardless of the interaction between heparin, insulin and bFGF, this study demonstrated that these three compounds in combination protect cells from high glucose or heparanase injury.

4. Heparanase Upregulation in High Glucose Treated Porcine Aortic Endothelial Cells is Prevented by Insulin and Heparin

4.1. Introduction

Microangiopathy and macroangiopathy, the complications of diabetes mellitus, are characterized by development of microvascular pathology in the retina and renal glomerulus as well as pathological changes typical of arterial disease in macrovasculature (Deckert *et al*., 1996; Gall *et al*., 1995; Stehouwer and Schaper, 1996). The endothelial cells lining all vessels appear to be the initial target in vascular damage by hyperglycemia. This endothelial injury is characterized by alteration in anticoagulant and profibrinolytic activity (Stehouwer, 1998), a decrease in nitric oxide-mediated vasodilation (Elliott *et al*., 1993), formation of advanced glycosylation end-products (Kirstein *et al*., 1990) and exaggerated proliferation of endothelial cells and thickening of the basement membrane (Fischer *et al*., 1979, Kefalides, 1981). These abnormalities not only result in vasoconstriction and narrowing of the blood vessel lumen, tissue ischemia, and eventually infarction, but also contribute directly to an increase in vascular permeability (Cowell and Lopes-Virella, 1988; Perejda, 1987).

Heparan sulfate (HS) is a glycosaminoglycan (GAG) that is associated with the cell membrane, basement membrane and extracellular matrix (ECM) (Kjellen and Lindahl, 1991). Depletion of HS and/or alteration in GAG metabolism may be a major mechanism of endothelial injury (Kanwar *et al*., 1980; Deckert *et al*., 1989). Previous studies showed that diabetic patients with a genetic defect in sulfotransferase, an enzyme required for GAG sulfation and needed for the regulation of endothelial HS production, were more likely to develop proteinuria and angiopathy (Gambaro and van der Woude, 2000). Other studies also showed decreased serum HS and increased urine GAGs in patients with overt diabetic nephropathy (Perlmutter *et al*., 1989, Shimomura and Spiro, 1982). The highly negatively charged HS, due to sulfate and carboxylate residues in its structure, controls the vascular permeability by acting as a “charge barrier” to the largely anionic plasma proteins (Hardebo and Kahlstrom, 1985). Although loss of HS is a major cause of endothelial injury in angiopathy induced by hyperglycemia, the cause of HS degradation is still unknown.

Heparanase, an endoglucuronidase, breaks down HS. It is involved in fundamental biological phenomena and pathological processes, such as inflammation, angiogenesis, autoimmunity and cancer metastasis (Vlodavsky and Friedmann, 2001). It is normally found in cytotrophoblasts, platelets, mast cells, neutrophils, macrophages, T and B lymphocytes and lymphoma (Vlodavsky *et al*., 1992). Heparanase may be induced by hyperglycemia and contribute to EC dysfunction by degradation of HS. Heparanase activity was

found in the urine and heparanase protein was expressed in renal glomerular cells of some diabetic patients (Katz *et al*., 2002).

Evidence from previous studies showed that insulin and/or heparin prevented the formation of intercellular gaps of endothelial cells cultured in high glucose medium (Mandal *et al*., 2000). This suggests that insulin and/or heparin may play a role in protection of endothelial cells from high glucose injury by preventing heparanase upregulation. bFGF has high potential to increase proliferation of capillary endothelial cells *in vitro*, has high affinity for heparin or HSPG (Broadley *et al*., 1989) and may inhibit HSPG degradation and protect cells from injury. Thus, our objectives were to determine if heparanase was upregulated in endothelial cells exposed to high glucose and if heparin and/or insulin and/or bFGF protect cells from injury by suppressing heparanase expression.

4.2. Experimental Procedures

Endothelial Cell Cultures

Porcine aortic endothelial cells (PAECs) were cultured according to the method of Gotlieb and Spector (1981). Primary cultures of PAECs were obtained directly from the porcine aorta. The aorta segment was cut and washed three times in Ca^{2+}-Mg^{2+}-free Dulbecco's phosphate-buffered saline (CMF-DPBS) while connective tissue was trimmed from the outside of the aorta. The aorta was held in an upright position by clamping one end of the aorta with hemostats. The lumen of the aorta was rinsed three times with

CMF-DPBS and then filled with collagenase solution (Type IV, SIGMA, St. Louis, MO, USA; 1 mg/ml in CMF-DPBS) for six minutes. After removing the collagenase solution, the lumen was gently rinsed with M199 (GibcoBRL, Life Technologies, Inc., Grand Island, NY, USA) containing 5% fetal bovine serum (FBS, GibcoBRL, Life Technologies, Inc), 50 µg/ml penicillin and 10 µg/ml streptomycin. The medium in the aorta was removed and plated onto 60 mm tissue culture dishes which were incubated at 37°C with 5% CO_2/95% air in a humidified environment. PAECs were identified by their morphological appearance consisting of a monolayer of cobblestone-like flattened cells, and the presence of von Willebrand factor (vWF) in initial cultures. Non-endothelial-like cells, such as smooth muscle cells and fibroblasts were destroyed by mechanical suction before the first passage. In order to pass cells, confluent cultures were washed twice with sterile CMF-DPBS and cells were removed from the dish by addition of trypsin solution (0.025% with EDTA in CMF-DPBS) for two or three minutes at room temperature. The cells were resuspended in culture medium and transferred to 60 mm dishes for further passage. The cells were transferred to 35 mm dishes at passage four for experiments, except when initial cultures were used.

Reagents

Glucose, heparin, insulin, hydrogen peroxide and mannitol were first prepared as a stock solution in CMF-DPBS. Concentration of stock solutions for glucose (D-Glucose, BDH Inc. Toronto, Canada) was 3M (540 mg/ml),

bovine lung heparin (151 USP U/mg Upjohn Pharmaceuticals, Kalamazoo, MI, USA) was 0.1 mg/ml, insulin (Humulin® N) was 100 U/ml, hydrogen peroxide (H_2O_2, SIGMA) was 10 mM and mannitol (SIGMA) was 600 mM**.** bFGF (SIGMA) was prepared as a stock solution of 1 ng/µl, and then diluted to 0.1 ng/µl in M199 without serum.

Treatment of Cultured Cells

Cultured PAECs passage 4, in 35 mm dishes were treated with high glucose, glucose plus heparin, glucose plus insulin and glucose plus heparin plus insulin as well as glucose plus bFGF. For glucose addition, 10 µl of 3 M (540 mg/ml) glucose stock solution was added to 1 ml culture medium to give the final concentration of 30 mM (5.4 mg/ml). For heparin addition, 5 µl of 0.1 mg/ml heparin was added to 1 ml medium with glucose to give the final concentration 0.5 µg/ml. For insulin addition, 10 µl of 100 U/ml insulin was added to 1 ml medium with glucose to give a final concentration of 1 U/ml. The cells were also treated with glucose plus heparin plus insulin at concentrations used when added alone. For bFGF addition, 10 µl of 0.1 ng/µl or 1 ng/µl of bFGF was added to 1 ml cell medium with glucose to give a final concentration of 1 ng/ml or 10 ng/ml. Medium was changed and glucose, insulin, heparin and bFGF were added fresh every other day for seven days. Cells were also treated with 30 mM mannitol where 50 µl of 600 mM mannitol stock solution was added to 1 ml medium to give a final concentration of 30 mM. Medium was changed every other day for seven days with fresh

mannitol addition. For addition of H_2O_2, 10 µl of 10 mM H_2O_2 was added to 1 ml medium to give a final concentration of 0.1 mM for 24 hrs. Control passage four PAECs were also studied as well as initial confluent and non-confluent cultures.

Collection of Control Porcine Tissue

Samples of liver, aortic smooth muscle and kidney were collected from newly slaughtered healthy, untreated pigs. The 50 mg samples were stored in RNA stabilization reagent (QIAGEN, RN Later™, Mississauga, ON, Canada) at room temperature for three or four days.

Detection of Heparanase mRNA

In order to isolate RNA from cells with different treatment and porcine tissue, fresh cells in the dish and tissue from the RNA stabilization reagent were washed three times in ice cold CMF-DPBS. Then, total RNA was isolated according to the manufacturer procedures using RNeasy® Mini Kit (QIAGEN). The concentration of RNA was determined by ultraviolet absorption at 260 nm using a spectrophotometer (Cary 100 Bio UV-Visible). All RNA samples had spectrophotometric ratio of absorbances between 1.7-2.0 at the wavelengths OD_{260} and OD_{280} indicating pure RNA isolates. The RNA was visualized on a 1% formaldehyde agarose gel. Then 1 µg of total RNA was used to perform Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) using the kit of SupersCript™ One-Step RT-PCR with

Platinum® Taq (InVitrogen. Gibco. Life Technologies. Burlington, ON, Canada) and primers specific for rat heparanase gene. The specific primers Hep 458 5'-CAAGAACAGCACCTACTCA-3' and Hep 1055 5'-CACATAAAG CCAGCTGCA-3' were designed using Gene Fisher Program to amplify a 597 bp DNA fragment. The primers were designed to span intron regions in order to prevent the amplification of contaminating genomic DNA. As well, the internal standard house keeping gene, *β*-actin, amplified by using sense primer 5'-GGACTTCGAGACGGAGATGG-3' and anti-sense primer 5'-GCAC CGTGTTGGCGTAGAGG-3' for each RNA sample was expressed as a 233 bp cDNA. The RT-PCR profile initially consisted of a cDNA synthesis step of 30 minutes at 50°C followed by 5 minutes at 94°C to inactive the reverse transcriptase and activate the taq polymerase. The RT-PCR conditions were an initial denaturation at 94°C for 2 min, and 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 2 min. The RT-PCR products (10 µl) were run on a 1.5% agarose gel to distinguish the 597 bp cDNA fragment of heparanase gene and 233 bp cDNA for *β*-actin which were visualized by agarose gel within 0.5-1 µg/ml of ethidium bromide (SIGMA).

Detection of Heparanase Activity

To determine the ability of heparanase to break down HSPGs, ^{35}S-labeled ECM produced by bovine corneal endothelial cells (BCECs) were incubated with cell lysates from various treatments. Supernatants from the

incubation of lysates with ECM were analyzed for degradation of ^{35}S-labeled products (HS). The procedure was performed as described below.

Culture of bovine corneal endothelial cells (BCECs): The primary BCECs were obtained from newly slaughtered cow eyes. The cow eyes were rinsed with 75% ethanol to sterilize the outside of the corneas. A small hole was made by puncturing the crystal-clear corneas with a sterilized needle. Then the corneas were separated from the eye by using small scissors starting from the small hole. After the pieces of corneas were washed thoroughly with CMF-DPBS, they were put onto the bottom of culture dishes with the endothelial side up. The BCECs were gently scraped from the cornea by using a metal spatula and then transferred to a 60 mm culture dish containing 5 ml of BCEC medium [DMEM supplemented with 10% FBS, 5% calf serum (CS, Life Technologies, Inc.), 50 μg/ml gentamycin, and 0.25 μg/ml fungizone]. After these primary culture dishes were incubated at 37°C in a humidified incubator with a 5% CO_2/ 95% air environment for 5 days, the media was changed and 1 ng/ml bFGF (human recombinant, SIGMA) was added every other day. At day ten, the primary cells were trypsinized using 0.025% trypsin and suspended in BCEC medium, and then 2 ml of cell suspension were transferred into 60 mm gelatinized dishes. For gelatinizing dishes, a 0.2% w/v gelatin (Type B, Bovine Skin, SIGMA) solution in CMF-DPBS was autoclaved and filtered at 0.22 μm after the solution cooled. The gelatin solution (3 ml) was used to cover the 60 mm culture dish which was

stored at 4 °C for at least 3 hrs. Cell medium was changed and bFGF was added every other day.

Preparation of ^{35}S-labeled extracellular matrix (ECM): Passage two to four of BCECs (about 2×10^5 cells) were seeded on 35 mm culture dishes with BCEC growth media supplemented by 5% dextran (SIGMA). Then 40 µCi $Na_2{}^{35}SO_4$ (540 to 590 mCi/mmol, Amersham) was added to the cultures on days two and five without media change, although 0.5 ml of fresh media was added to each dish on day five. When the cells became confluent, ten to 12 days after seeding, cell medium was removed and the cells were washed three times with CMF-DPBS, and then exposed to the cell lysis solution (0.5% Triton X-100 and 0.025 N NH_4OH in CMF-DPBS) for three to four minutes with gentle shaking at room temperature followed by washing three times with CMF-DPBS. The dishes were examined under an inverted phase microscope, where the extracellular matrix (ECM) appeared as a delicate uniform network and remained firmly attached to the plastic dishes and free of cellular debris and nuclei.

Measurement of heparanase using ^{35}S-labeled extracellular matrix (ECM): Cultured PAECs were washed twice with CMF-DPBS and dissociated with 0.025% trypsin, suspended in culture medium, and the cell numbers were counted. After washing twice with CMF-DPBS, the cells ($5\text{-}7\times10^5$) were resuspended in 1 ml phosphate buffer, pH 6.5 with 1 mM Mg^{+2},

and lysed by three cycles of freezing at -80°C and thawing at 37°C. The cell lysates were incubated with newly made ^{35}S-labeled ECM for 24 hrs at 37°C. To evaluate the occurrence of HS degradation, 0.8 ml aliquot of the supernatant was applied to Sepharose 6B columns (0.9×8 cm) and gel filtered. Fractions of 200 µl were eluted with CMF-DPBS at a flow rate of 6 ml/hr and counted for radioactivity by a *β*-scintillation counter (BECKMAN, LS6000IC). The high-Mr ^{35}S-labeled material released from ECM was at peak I after V_0 (Kav<0.2) and the degraded low-Mr ^{35}S-labeled products was at peak II (0.5<Kav<0.8) according to previous studies (Matzner *et al*., 1985). Thus, intact HS should be eluted at peak I (fraction 5-10) and degraded HS at peak II (fraction 15-25).

4.3. Results

Heparanase mRNA Expression

To determine if heparanase mRNA is detectable in control untrated EC cultures, total RNA was isolated from initial confluent and non-confluent PAECs. Further, to determine if other cells derived from the porcine model produced heparanase mRNA, total RNA was isolated from untreated porcine tissue including porcine liver, kidney, and aortic smooth muscle. RT-PCR (10 µl) products of each sample run on agarose gel electrophoresis, were negative for heparanase mRNA, as no bands appeared at 597 bp (Figure 4.1 and Figure 4.2).

To determine if heparanase mRNA is detectable in high glucose treated PAECs, total RNA was isolated from PAECs treated with high glucose. RT-PCR products, run on agarose gel, showed a positive band appearing at 597 bp in high glucose (30mM) treated PAECs (Figure 4.3). To determine if insulin and/or heparin inhibited heparanase production induced by high glucose, total mRNA was isolated from control PAECs and PAECs treated with glucose plus insulin, glucose plus heparin and glucose plus insulin plus heparin. After running RT-PCR, there were no bands showing at 597 bp (Figure 4.3).

To determine if free radical production and changes in osmolarity induce heparanase expression, total RNA was isolated from PAECs treated with H_2O_2 and with mannitol. RT-PCR products run on agarose gel electrophoresis showed that a positive band at 597 bp appeared in H_2O_2 treated PAECs, but not in mannitol treated cells (Figure 4.4).

To determine if bFGF inhibits heparanase mRNA expression, total RNA was isolated from PAECs treated with high glucose and bFGF. RT-PCR products run on agarose gel electrophoresis showed that a positive band at 597 bp in PAECs treated with 1 ng/ml or 10 ng/ml of bFGF and high glucose (Figure 4.5) indicating that bFGF likely does not prevent heparanase expression in high glucose treated cells.

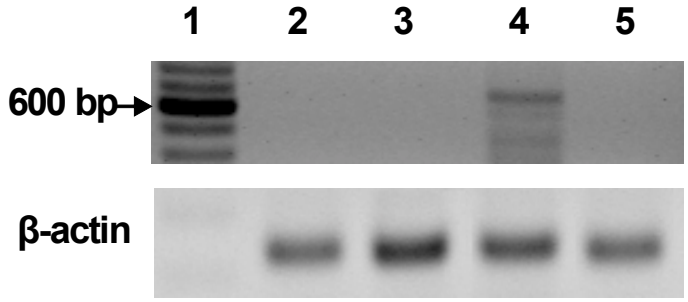


Figure 4.1. Heparanase mRNA was not Detectable in Fresh Untreated Porcine Tissue

Samples of liver, kidney and aortic smooth muscle were obtained from fresh porcine tissue. Each sample (50 mg) was stored in RNA stabilization reagent at room temperature for three or four days and then total tissue RNA was extracted. Lane 1: DNA ladder, Lane 2: porcine liver, Lane 3: porcine kidney, Lane 4: glucose treated PAECs used as positive control, Lane 5: porcine aortic smooth muscle.

Total RNA was isolated from PAECs and fresh porcine liver, kidney and aortic smooth muscle with RNeasy extraction Kit from QIAGEN. Total RNA (1μg) was run on agarose gel (1.5% containing 0.5-1 μg/ml ethidium bromide). *β*-actin mRNA expression was used as an internal control. Heparanase mRNA was expressed as a 597 bp cDNA close to 600 bp in the DNA ladder.

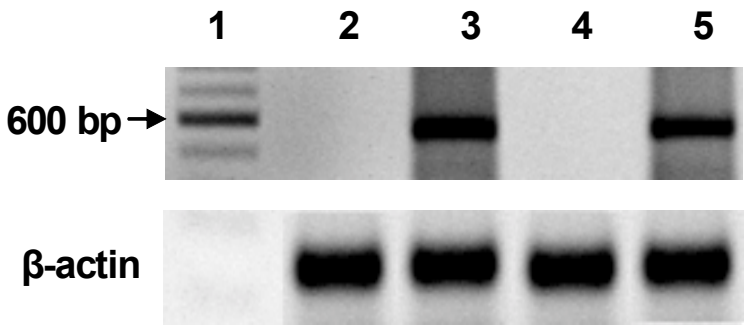


Figure 4.2. Heparanase mRNA was not Detectable in Primary Non-confluent and Confluent PAECs

Primary non-confluent (day 8) and confluent (day 15) cultured PAECs were used. Confluent primary PAECs treated with high glucose (30mM) for seven days, and then total RNA was extracted. Lane 1: DNA ladder, Lane 2: primary non-confluent, Lane 3: primary confluent treated with high glucose, Lane 4: primary confluent, Lane 5: high glucose treated passage 4 PAECs used as a positive control.

Total RNA was isolated from non-confluent and confluent PAECs with RNeasy extraction Kit from QIAGEN. Total RNA (1μg) was run on agarose gel (1.5% containing 0.5-1 μg/ml ethidium bromide). *β*-actin mRNA expression was used as an internal control. Heparanase mRNA was expressed as a 597 bp cDNA close to 600 bp in the DNA ladder.

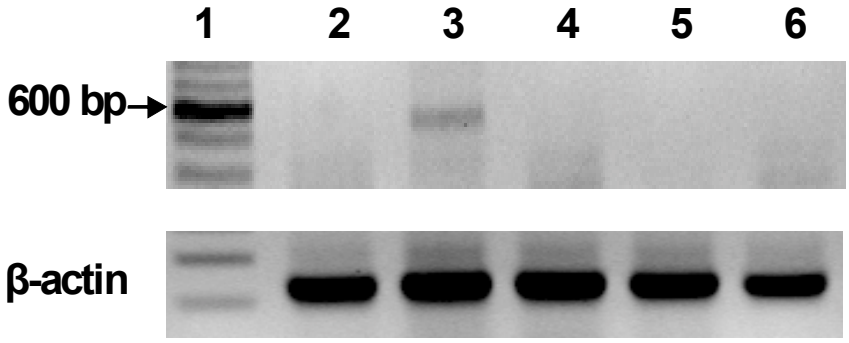


Figure 4.3. High Glucose Induced Heparanase mRNA which was Inhibited by Insulin and/or Heparin

Confluent (passage 4) PAECs were treated as follows: Lane 2: control, Lane 3: high glucose (30 mM), Lane 4: high glucose plus heparin (0.5 μg/ml), Lane 5: high glucose plus insulin (1 U/ml), Lane 6: high glucose plus heparin plus insulin for seven days. Medium was changed and reagents were added fresh every other day. Total RNA was extracted 48 hours after the last addition of reagents. Lane 1: DNA ladder.

Total RNA was isolated from PAECs treated high glucose and/or heparin and/or insulin and control cells with RNeasy extraction Kit from QIAGEN. Total RNA (1μg) was run on agarose gel (1.5% containing 0.5-1 μg/ml ethidium bromide). *β*-actin mRNA expression was used as an internal control. Heparanase mRNA was expressed as a 597 bp cDNA close to 600 bp in the DNA ladder.

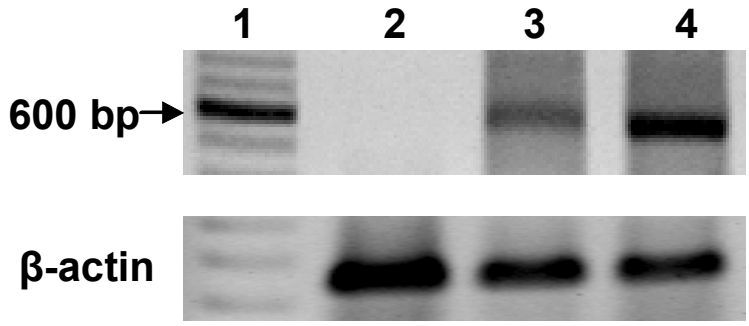


Figure 4.4. Heparanase mRNA was Induced by H_2O_2, but not Mannitol

Confluent PAECs (passage 4) were treated with mannitol (30 mM) for seven days. Medium was changed and fresh mannitol added every other day. Total RNA was extracted 48 hours after last mannitol addition. Confluent PAECs (passage 4) were treated with H_2O_2 (0.1 mM) for 24 hours and then total RNA was extracted. Lane 1: DNA ladder, Lane 2: mannitol, Lane 3: H_2O_2, Lane 4: high glucose as a positive control.

Total RNA was isolated from PAECs treated with H_2O_2 or mannitol with RNeasy extraction Kit from QIAGEN. Total RNA (1µg) was run on agarose gel (1.5% containing 0.5-1 µg/ml ethidium bromide). *β*-actin mRNA expression was used as an internal control. Heparanase mRNA was expressed as a 597 bp cDNA close to 600 bp in the DNA ladder.

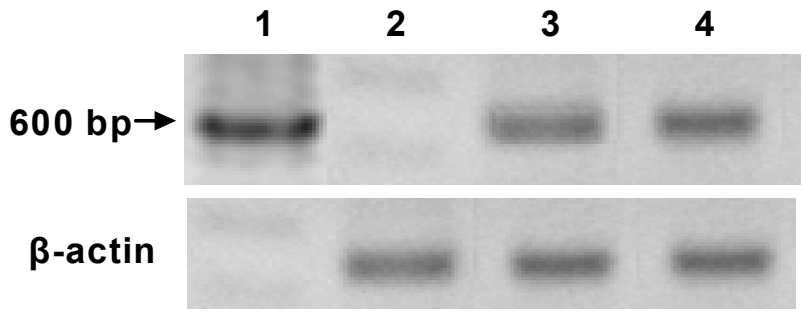


Figure 4.5. Heparanase mRNA was Expressed in High Glucose and bFGF Treated PAECs

Confluent PAECs (passage 4) were treated with high glucose (30 mM) and bFGF (1 ng/ml and 10 ng/ml) for seven days. Medium was changed and fresh glucose and bFGF added every other day. Total RNA was extracted 48 hours after last addition of glucose and bFGF. Lane 1: DNA ladder, Lane 2: control, Lane 3: glucose plus bFGF (1 ng/ml), Lane 4: glucose plus bFGF (10 ng/ml).

Total RNA was isolated from PAECs treated with high glucose and bFGF with RNeasy extraction Kit from QIAGEN. Total RNA (1µg) was run on agarose gel (1.5% containing 0.5-1 µg/ml ethidium bromide). *β*-actin mRNA expression was used as an internal control. Heparanase mRNA was expressed as a 597 bp cDNA close to 600 bp in the DNA ladder.

Heparanase Activity Assay

In Figure 4.6 and 4.7 are shown recovery of intact HS degaded HS following treatment of ECM with lysates of ECs exposed to various treatments. Incubation of ^{35}S-labeled ECM with high glucose or H_2O_2 treated PAEC lysates resulted in release of low-Mr ^{35}S-labeled degradation products into the incubation medium at peak II (0.5<Kav<0.8, fraction 15-25). Results were similar when the ECM was treated with 0.1 U/ml heparinase I in phosphate buffer (positive control) shown in Figure 4.7. Addition of PAECs lysates from cells treated with insulin and/or heparin as well as high glucose to the ECM did not show the release of low-Mr degradation products, but showed high-Mr ^{35}S-labeled products at peak I (Kav<0.2, fraction 5-10) shown in Figure 4.6. Lysates from mannitol treated PAECs showed high-Mr ^{35}S-labeled products at peak I with no degradation products found at peak II shown in Figure 4.7. Cell lysates in DPBS from control untreated PAECs showed no HS degradation products at peak I (negative control) shown in Figure 4.7.

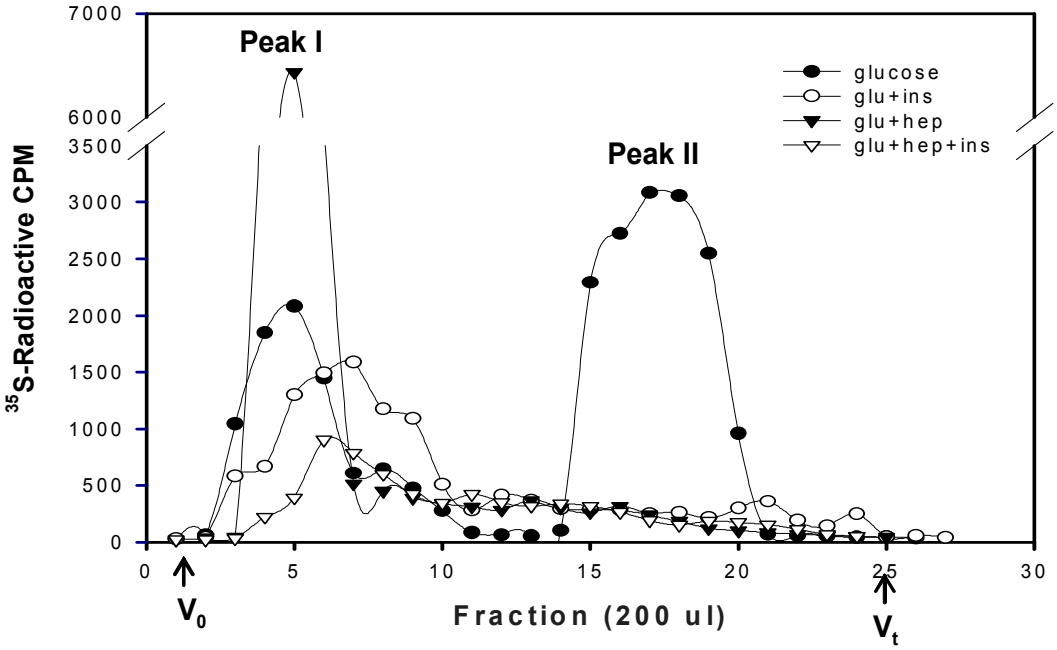


Figure 4.6. High Glucose Induced Heparanase Activity which was Inhibited by Insulin and/or Heparin

Heparanase activity was expressed in high glucose (30 mM) treated PAECs (●) shown at peak II, but not in high glucose +heparin (0.5 μg/ml) (▼), high glucose +insulin (1U/ml) (○), and high glucose + heparin + insulin (Δ) treated cells, shown at peak I. (V_0: void volum, V_t: total volum)

^{35}S-labeled ECM was produced by incubating bovine corneal endothelial cells (passage two to four) in 35 mm culture dishes with $Na_2{}^{35}SO_4$ (80 μCi/ml). Confluent cells were removed with a lystic solution (0.5% Triton X-100 and 0.025 N NH_4OH in CMF-DPBS), leaving the ECM firmly attached to the dishes.

PAECs (passage 4) were treated with high glucose and heparin and/or insulin for seven days or H_2O_2 for 24 hrs. Cells were lysed by three cycles of freezing at -80ºC and thawing at 37 ºC in pH 6.5 phosphate buffer with 1 mM Mg^{2+}. PAECs lysate (1 ml) was incubated with ^{35}S-labeled ECM at 37ºC for 24 hrs. The incubation supernatant (0.8 ml) was gel filtered in Sepharose 6B columns. Fractions (200 μl) were eluted with CMF-DPBS at a flow rate of 6 ml/hr and radioactivity by a β-scintillation counter. Heparanase activity was detected by elution of degraded HS at peak II. Non-degraded HS eluted at peak I showed no heparanase activity.

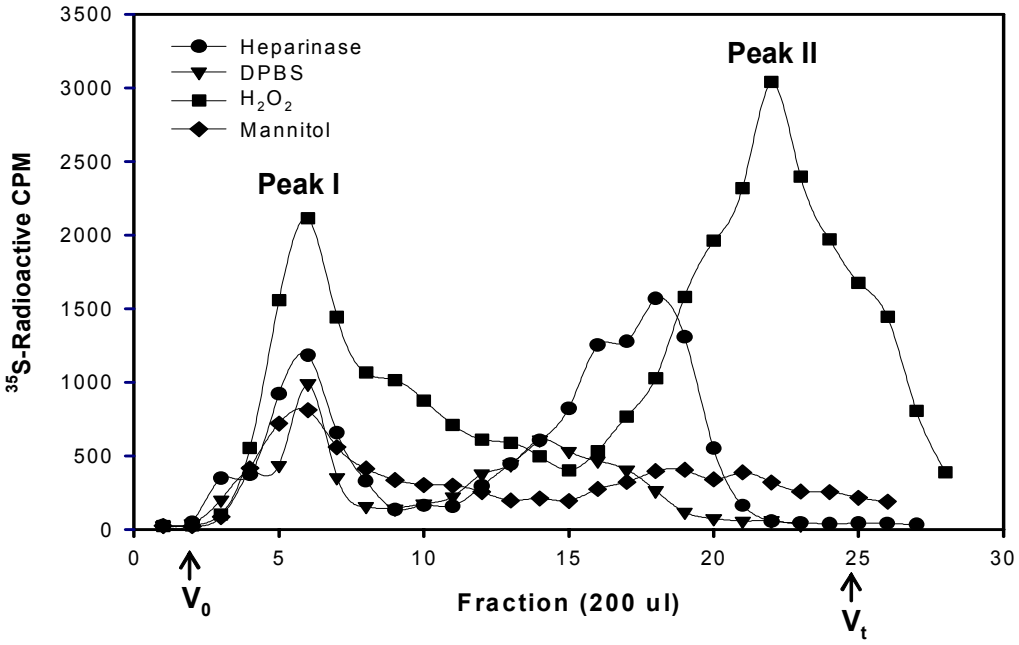


Figure 4.7. Heparanase Activity was Present in H_2O_2 but not in Mannitol Treated PAECs.

ECM incubated with heparinase I (0.1 U/ml) (positive control) (●) and CMF-DPBS (negative contol) (▼) are showed at peak II and at peak I respectively. Heparanase activity was expressed in H_2O_2 (0.1 mM) treated PAECs (■) shown at peak II, but not in mannitol (30 mM) preated PAECs (♦), shown at peak I. (V_0: void volum, V_t: total volum)

^{35}S-labeled ECM was produced by incubating bovine corneal endothelial cells (passage two to four) in 35 mm culture dishes with $Na_2{}^{35}SO_4$ (80 µCi/ml). Confluent cells were removed with a lystic solution (0.5% Triton X-100 and 0.025 N NH_4OH in CMF-DPBS), leaving the ECM firmly attached to the dishes.

PAECs (passage 4) were treated with mannitol for seven days or H_2O_2 for 24 hrs. Cells were lysed by three cycles of freezing at -80ºC and thawing at 37ºC in pH 6.5 phosphate buffer with 1 mM Mg^{2+}. PAECs lysate (1 ml) was incubated with ^{35}S-labeled ECM at 37ºC for 24 hrs. The incubation supernatant (0.8 ml) was gel filtered in Sepharose 6B columns. Fractions (200 µl) were eluted with CMF-DPBS at a flow rate of 6 ml/hr and radioactivity by a β-scintillation counter. Heparanase activity was detected by elution of degraded HS at peak II. Non-degraded HS eluted at peak I showed no heparanase activity.

4.4. Discussion

The monolayer of endothelial cells (ECs) are the major metabolically active cells lining all blood and lymphatic vessels and play an important role in the structural integrity and normal function in the circulatory system. Heparan sulfate proteoglycans (HSPGs) are synthesized by vascular ECs and incorporated into their surface, ECM and basement membrane. The HSPG macromolecules consist of a protein core to which several polysaccharide chains called GAGs are covalently attached (Muir, 1977). The GAGs are highly negatively charged due to sulfate and carboxylate residues present in their structure (Bernfield *et al*., 1999). The negative charges on the luminal surface of the vascular vessel wall are thought to play a major role in controlling vascular permeability by acting as “a charge barrier” to the transvascular movement of the largely anionic plasma proteins such as albumin (Hardebo and Kahlstrom, 1985). Previous studies have shown that HS specifically interacts with various adhesive macromolecules such as fibronectin, laminin and collagen, and hence its degradation may disassemble the ECM and basement membrane, resulting in EC detachment and damage to vessel wall integrity (Kraemer, 1971).

Heparanase is an endo-*β*-D-glucuronidase that breaks down the GAG chains of HSPG at specific sites, and can act on the endothelial cell surface, ECM and basement membrane. Some human and animal heparanase genes were recently cloned and heparanase proteins were encoded by the genes, however, the porcine heparanase gene and protein

has not been previously studied. Alignment of the human, mouse and rat heparanase amino acid sequences corresponding to the 50-KDa human mature enzyme reveal 80-93% identity (Vlodavsky *et al*., 1999; Hulett *et al*., 1999). Therefore, the rat heparanase gene was selected to design the primers to amplify the heparanase cDNA for porcine tissue and PAECs in our studies. There was no heparanase mRNA expression in fresh untreated porcine tissue and initial primary confluent and non-confluent PAEC cultures (Figure 4.1 and Figure 4.2). This is similar to what has been demonstrated in humans (Godder *et al*., 1991). Thus, the PAECs can be used as an in vitro model to study heparanase expression in ECs for human disease.

It was reported that heparanase is rarely found in intact original human ECs and their lysates or in bovine aortic ECs following exposure to various physiological activators such as bFGF, thrombin, endotoxin, interleukin-1 (Il-1), TNF, calcium ionophore, or radiation (Godder *et al*., 1991; Nakajima *et al*., 1986). This finding suggests that ECs contain a heparanase that is released only from injured ECs that may then degrade HSPG. HSPG degradation involves vessel wall disruption and new vessel formation both in normal and pathological situations such as wound healing, tissue repair and cancer progression (Moscatelli *et al*., 1985; 1986). Our present study is the first to show that heparanase mRNA expression is found in PAECs treated with high glucose and H_2O_2 (Figure 4.3 and Figure 4.4). PAECs injury was further demonstrated by a decrease in numbers of live cell /culture and increase in lactate dehydrogenase (LDH) release in cell medium in high

glucose treated cells compared to control cells (Mandal *et al*., 2002). Heparanase activity was found in PAECs treated with high glucose and H_2O_2 (Figure 4.5 and Figure 4.6) in agreement with the finding of heparanase mRNA expressed in PAECs treated with high glucose and H_2O_2. Heparanase expression and degradation of HS in high glucose treated PAECs are likely to contribute to EC injury. An increase in osmolarity is likely not the cause of high glucose injury and heparanase upregulation since no expression of heparanase mRNA and protein was found in mannitol treated cells (Figure 4.4 and Figure 4.6). Heparanase expressed in H_2O_2 treated cells indicates that the production of free radicals may be one of the mechanisms in the pathway by which hyperglycemia induces endothelial injury and upregulation of heparanase. Previous studies found decreased serum HS and increased urine GAGs in patients with overt diabetic nephropathy (Yokoyama *et al*., 1999, Perez-Blanco *et al*., 2000), and less GAG content in glomerular basement membrane of the patients with diabetic nephropathy compared to nondiabetic control subjects (Parthasarathy and Spiro, 1982). In patients with diabetic nephropathy, the decreased HSPG in the glomerular basement membrane correlated with the degree of proteinuria (Tamsma *et al*., 1994). As well, changes in content and structure of HSPG in the glomerular basement membrane were thought to be associated with proteinuria in patients with diabetic nephropathy (Rosenzweig and Kanwar, 1982). Increased glomerular permeability to largely anionic plasma proteins may result from loss of HSPG, the negative “charge barrier” (Kanwar *et al*.,

1980) due to HS degradation by heparanase. Heparanase activity was shown in renal glomerular cells and in the urine of diabetic patients (Katz *et al*., 2002). In addition, a decrease in HS content in the aortic intima of diabetic patients has been observed (Wasty *et al*., 1993), suggesting that the abnormalities in HS metabolism could occur in the whole cardiovascular system and not only in the kidney, indicating association between cardiovascular complications and nephropathy. However, it is still unknown where heparanase is produced in the vasculature.

The capillary ECs, injured by high glucose in the glomerulus, may induce heparanase upregulation. Heparanase expressed in ECs in both large and small vessels may play an important role in EC dysfunction. ECs in macro and micro vessels have different properties, but both are characterized by the same pathological features in diabetes mellitus. In both macro and micro vessels, exaggerated proliferation of ECs and thickening of the basement membrane result in narrowing of the vessel lumen which contributes to premature thrombosis, and ischemia (Perejda *et al*., 1987, Richardson *et al*., 1980; Colwell and Lopes-Virella, 1988). Heparanase upregulation in injured ECs could be a common factor for pathological effects presented in both endothelial cells from both large and small vessels.

Heparin is intracellularly located in the mast cells and released as a GAG. Like HS, heparin controls vascular permeability by forming a negative “charge barrier” on the luminal surface of endothelium (Muir, 1977). In addition, exogenous heparin is used as an anticoagulant agent and

ameliorates increased vascular permeability caused by various polycationic substances (Fairman *et al*., 1987; Peterson *et al*., 1987). The ability of heparin to protect endothelium has been shown in several studies. Heparin accumulates in endothelium against a concentration gradient (Hiebert *et al*., 1993). Heparin treatment results in increased formation of HS in cultured EC surfaces (Nader *et al*., 1991) and protects cultured ECs from injury when exposed to free radicals (Hiebert and Liu, 1990). Our studies support the idea that heparin is protective and prevents the upregulation of heparanase in high glucose injured endothelium. Insulin regulates glucose metabolism and promotes glucose up-take and utilization to reduce the glucose concentration in hyperglycemia. Heparin, insulin and a combination of insulin and heparin prevent the intercellular gaps in ECs exposed to high glucose (Mandal *et al*., 2000). Similar results obtained from our experiment showed an increase in live cell number and decreased LDH release in PAECs treated with high glucose plus insulin and/or heparin compared to high glucose alone (Mandal *et al*., 2002). Our results in the present study, show no expression of heparanase mRNA and activity in high glucose plus insulin and/or heparin treated cells, agreeing with the protective effects demonstrated in previous studies, and also support the idea that GAGs and heparin-like polyanionic molecules inhibit heparanase upregulation (Bar-Ner *et al*., 1987). This study further demonstrates, at the gene level, inhibition of EC heparanase expression in high glucose treated cells by insulin and/or heparin, but not bFGF, even though bFGF has the ability to enhance cell proliferation.

In summary, diabetic vascular complications are primarily caused by endothelial injury. Degradation of HSPGs contributes to endothelial cell injury resulting in loss of anionic charges in the vasculature and increasing vascular permeability as well as other pathological effects. Our studies confirm that heparanase over-expression is associated with endothelial injury and could contribute to the diabetic vascular complications. Furthermore, insulin and heparin, alone or in combination, protect ECs from injury through down-regulation of heparanase expression induced by high glucose. These results provide the theoretical background for further studies in the field of pharmacological intervention in diabetic vascular disease.

5. GENERAL DISCUSSION

Endothelial dysfunction induced by hyperglycemia is believed to be a pivotal stage in diabetic cardiovascular complications which is the main causes of morbidity and mortality in diabetes mellitus. A considerable body of evidence has shown that many metabolic abnormalities and by-products are involved in the pathways of endothelial dysfunction. In the present study, in order to mimic hyperglycaemia cultured aortic endothelial cells (ECs) were treated with high concentrations of glucose (30 mM). Treatment of cells with high glucose cause damage to ECs shown by a decrease in live cell number and increase in LDH release which is in agreement with previous evidence (Lorenzi, 1992). When Heparinase I was added to ECs cultured in serum free medium, EC damage was similar to that seen in high glucose treatment. These data suggest upregulation of heparanase in the diabetic condition may play a role in endothelial dysfunction. However, it is likely that EC injury caused by high glucose is more complicated than heparanase upregulation alone and many mechanisms in addition to heparanase induction are likely involved when ECs are injured by high glucose. Heparanase mRNA and activity, expressed in cells treated with high glucose, indicate that high glucose indeed induces heparanase over-expression and heparanase is an additional contributing factor in cells injured by high glucose. Katz *et al*.,

(2002) identified that heparanase activity was found in both the glomerular mesangial and epithelial cells and in the urine of diabetic patients. As of now there have been no studies relating high glucose to upregulation of heparanase in endothelial cells. Heparanase is rarely expressed in intact original ECs and their lysates shown in studies with human and animal cells exposed to physiological stimuli (Godder *et al*., 1991). Our present studies showed that heparanase mRNA was not expressed in primary cultured control and passage 4 control porcine aortic ECs further confirming the above results. In addition we observed that heparanase mRNA was not expressed in fresh untreated porcine tissue such as liver, kidney, and aortic smooth muscle. Since heparanase is not normally expressed in human tissue, porcine ECs can be safely used as an *in vitro* model to study heparanase expression for human vascular diseases. Our studies are the first to show that heparanase mRNA and activity were expressed in ECs treated with high glucose suggesting EC injury in hyperglycemia, caused by upregulation of heparanase, contributes to diabetic vascular complications.

Since heparanase over-expression in ECs under hyperglycemic conditions is demonstrated in our study, the degradation of HSPG is likely involved in EC injury in diabetic vascular complications. Heparanase breaks down HSPG on the cell surface and in the ECM resulting in the release of enzymes, plasma proteins and growth factors such as bFGF and causing EC damage and vessel wall destruction and dysfunction. Loss of bFGF affects DNA synthesis and the processes of cell proliferation, replication and

differentiation. HSPG can act both as reservoirs of growth, differentiation and migration of many cell types (Folkman and Klagsbru, 1987). It is known that bFGF exerts its function only in the presence of HS through the formation of a bFGF/HS/bFGFR complex. Studies with cultured cells, deficient in cell surface HS, showed that the cells failed to proliferate in the presence of HS dependent growth factors even at significantly high concentrations of these growth factors. (Walker *et al*., 1994; Fannon and Nugent, 1996). Our results showed that bFGF alone could not protect cells from high glucose or heparinase I injury, but bFGF and heparin could protect cells from heparinase I injury in agreement with these findings. In addition, the result of heparanase mRNA expression in bFGF and high glucose treated ECs indicates that bFGF alone (even at doses as high as 10 ng/ml) could not inhibit the upregulation of heparanase induced by high glucose, further confirming the above findings. In the presence of heparin, bFGF protected cells from high glucose or heparinase I injury showed by increased live cell number and decreased LDH release as well as prevention of the expression of heparanase mRNA and activity compared to high glucose alone treated ECs. This suggests that heparin has the ability to ensure the physiological function of bFGF by inhibiting heparanase production either through promoting HS synthesis or reducing the degradation of HS. This result is also consistant with the idea that GAGs and heparin-like polyanionic molecules inhibit heparanase upregulation (Bar-Ner *et al*., 1987).

As we know, insulin is capable to enhancing cell proliferation by acting as a growth factor which is also demonstrated in our study on ECs treated with insulin alone. Addition of insulin to cell medium protected ECs from high glucose or heparinase I injury directly shown by a significantly increased live cell number and decreased LDH release compared to high glucose or heparinase I treatment alone. The combination of insulin and heparin protected ECs injured by high glucose or heparinase I even further shown by the highest live cell number and significantly decreased LDH release in each experiment. Insulin alone or in combination with heparin inhibited the up-regulation of heparanase demonstrated by the lack of heparanase mRNA and activity expressed in cells treated with high glucose and insulin and/or heparin. These findings agree with the observation that insulin and/or heparin prevented the intercellular gaps of ECs exposed to high glucose (Mandal *et al*., 2000). In the presence of bFGF, insulin and heparin also possessed the same protective effects on ECs injured by high glucose or heparinase I.

However, the exact mechanisms underlying insulin's protective effect on ECs injured by hyperglycemia are poorly understood. It has been shown that insulin-stimulated glucose uptake and insulin-stimulated endothelial function are regulated by similar signalling pathways (Rask-Madsen *et al*., 2001; Krook *et al*., 1997). The endothelial derived NO could play a role in these common insulin pathways as insulin can increase both cell proliferation and NO production in cultured ECs. NO is able to protect the vasculature

from dysfunction through its ability to regulate vascular tone and blood pressure and to reduce the production of reactive oxygen species (ROS) (Shen *et al*., 1994). Increased ROS, such as H_2O_2, may contribute to heparanase upregulation caused by high glucose. Our results showing heparanase mRNA and activity expressed in cells treated with H_2O_2 indicates that the production of ROS in hyperglycemia may induce the expression of heparanase. Previous studies show high glucose-derived induction of oxidative stress in human endothelial cells and porcine aortic vascular smooth muscle cells (Ceriello *et al*., 1992; Sharpe *et al*., 1998). It is plausible that the induction of heparanase by hyperglycemia is mediated by the production of ROS. Our results showing that heparanase induction by high glucose was inhibited by insulin, demonstrated by no heparanase mRNA and activity expressed in high glucose and insulin treated cells, suggest that increased NO production by insulin may suppress hyperglycemic oxidative stress, and thus inhibit heparanase upregulation. Since heparin also has the ability to protect cells from oxidative damage, the combination of insulin and heparin protecting cells from heparanase injury induced by high glucose could represent the common mechanism linking heparin and insulin in the same pathway related to the amelioration of oxidative stress and inhibition of heparanase upregulation.

In addition, an increase in osmolarity is likely not the cause of ECs injured by high glucose since heparanase mRNA and activity was not expressed in mannitol treated cells in the present study and no decrease in

cell proliferation and increase in cell apoptosis was shown by mannitol treatment in other studies (Ortiz *et al*., 1997).

In conclusion, our results from both cellular and molecular studies demonstrated that hyperglycemia is a critical factor contributing to endothelial injury which is in agreement with previous studies. For the first time we have determined that heparanase is induced by hyperglycemia and likely degrades HSPG suggesting this fundamental mechanism of EC injury in diabetic vascular complications. Even though the pathways or mechanisms involved in the protection on EC injury by insulin and/or heparin and/or bFGF may be quite variable, the combination of two or of three compounds exerts synergistically protective effects on EC injury under high glucose conditions. These results may shed some light on the mechanisms involved and on innovation of therapeutic drugs for human diseases associated with heparanase production such as diabetes mellitus and cancer.

6. CONCLUSION

- Porcine aortic endothelial cells can be used as an *in vitro* model to study heparanase expression as heparanase mRNA was not shown in control primary cultured confluent and non-confluent PAECs or in fresh untreated porcine tissue such as liver, kidney and aortic smooth muscle.

- Hyperglycemia is a cause of endothelial cell injury as live cell number decreased and LDH release increased in cells treated with high glucose concentrations which mimic hyperglycemia.

- Heparanase induction represents a mechanism of endothelial injury in hyperglycemia as heparanase mRNA and activity was expressed in ECs treated with high glucose. Also heparinase I damaged cultured ECs in serum free conditions.

- The production of reactive oxygen species may be involved in the mechanism of heparanase upregulation since heparanase mRNA and activity was expressed in ECs treated with H_2O_2.

- Cells injury in high glucose treated cultures was not due to changes in osmolarity since heparanase mRNA and activity was not expressed in ECs treated with equally high concentrations of mannitol.

- Insulin and heparin alone or in combination protect cells from high glucose or heparinase I injury and inhibit heparanase mRNA expression and activity.

- bFGF alone cannot protect cells from high glucose or heparinase I injury and cannot inhibit heparanase mRNA expression.

- The combination of heparin, insulin and bFGF can protect cells from high glucose and heparinase I injury.

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